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Exploring and Exploiting the Protein S100A7 as a New Target for Breast Cancer Therapy

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14. ABSTRACT
The following is a brief summary of the most significant achievement described herein:
The conditions and mechanisms regulating S100A7 expression in breast cancer cells are not well understood. Because inflammation can regulate S100A7 in skin cells, we treated breast tumor cells with several inflammatory cytokines, including oncostatin-M (OSM), interleukin-6 (IL-6), TNF-α, and IL-1β. Each of these could enhance S100A7 protein levels; however, OSM was by far the strongest inducer of S100A7 in breast cells and induced S100A7 at both the mRNA and protein levels. We noted that signaling by STAT3, PI3K and ERK1/2 were all necessary for OSM-induction of S100A7. The enhanced migratory rate bestowed by OSM stimulation was found to be dependent on S100A7. Finally, we observed that expression of S100A7 and OSM (and its receptor OSMR) were correlated in vivo, and that S100A7 may be correlated with poor patient prognosis only in the context of breast cancers that express OSMR.

15. SUBJECT TERMS
S100A7, breast cancer, inflammation, small molecules

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Introduction

The S100A7-Jab1 pathway in breast cancer

First associated with the abnormally differentiating keratinocytes of psoriatic lesions, S100A7 (psoriasin) has since been identified as an upregulated protein in a variety of epithelial tumor types. In normal epithelia of the skin and breast, S100A7 expression is generally weak or absent, but is induced in cells of dysplastic lesions. S100A7 is particularly associated with carcinoma *in situ* of skin and breast, and can be amongst the most highly expressed genes in breast carcinoma cells (1). S100A7 expression in breast cancer is associated with aggressive tumor types and poor patient prognosis (2, 3). Although S100A7 is an intracellular and secreted protein, investigation of its roles in breast cancer has focused solely on the intracellular component. In the cell, S100A7 interacts with the multifunctional protein Jab1 (c-jun activation domain binding protein 1, also known as CSN5) to influence several molecular pathways of breast cancer cells. Among the effects of this interaction are the nuclear accumulation of Jab1, stimulation of AP-1 transcriptional activity, activation of the pro-survival pathway NFκB/Akt, and degradation of the cell cycle inhibitor p27 (4, 5). Together, these effects contribute to increased cell viability in response to a variety of stressful stimuli, including loss of substrate adhesion, serum deprivation, and exposure to genotoxic agents. The S100A7-Jab1 interaction promotes changes in cell growth, adhesion, and invasiveness, enhances breast tumor progression *in vivo* (using nude mouse xenograft models) and is thus a functionally relevant pathway in breast cancer (4-6). Our lab has previously generated a S100A7 triple mutant (Asp56Gly, Leu78Met, Gln90Lys) that is deficient in its ability to bind Jab1. Breast cancer cells expressing the S100A7 triple mutant show a corresponding loss of the biological effects associated with S100A7 expression, and are significantly less tumorigenic *in vivo* (6). Therefore, we propose that disruption of this pathway may be a viable strategy for the treatment of S100A7(+) breast cancers.

Extracellular S100A7, inflammation, and the breast tumor microenvironment

It has been known for over a decade that secreted, extracellular psoriasin may have a role in modulating the immune response to tissue (particularly skin) damage. In 1996, it was reported that S100A7 can function as a potent chemotactic factor for CD4^{+} T lymphocytes and neutrophils (7). More recently, attention has focused on the antibacterial, defensin-like properties of S100A7 secreted by wounded skin (8, 9). Furthermore, inflammatory cytokines such as IL (interleukin)-22 and oncostatin-M (OSM) have been demonstrated to induce expression of S100A7 in human keratinocytes (10, 11). Importantly, we have observed in preliminary experiments that stimulation of breast cell lines with OSM can induce or enhance S100A7 expression (unpublished data). Thus, we have evidence to suggest that the link between S100A7 and skin inflammation may also be relevant to the biology of breast cancer. Despite our growing knowledge of the roles and regulation of S100A7 in the immune responses of skin, and while S100A7 is a known tumor promoter modulating intracellular signals in the breast, nothing is known of the role(s) of secreted S100A7 in the breast tumor microenvironment.

S100A7 expression has been repeatedly associated with inflammation in breast tumors. However, the types of infiltrating leukocytes and their various phenotypes have never been characterized. Intriguingly, two other proteins within the S100 protein family (S100A8/A9) are gaining increasing attention for their suspected roles in mediating cancer-associated immune responses (12). Breast tumors are often characterized by considerable leukocytic/inflammatory infiltrates, and can engage in tumor-promoting cytokine cross-talk activity with such stromal

leukocytes. For example, the breast cancer cell lines MDA-MB-231 and T47D have been shown to induce OSM production in neutrophils isolated from whole blood, which in turn stimulates cell detachment, invasive capacity, and production of vascular endothelial growth factor by the tumor cells (13). In transgenic mouse models, tumor-associated macrophages have been shown to be important for initiation of the angiogenic switch in pre-malignant breast tumors (14). Based on such studies, the concept that inflammatory processes can significantly impact cancer development is gaining attention (15). Therefore, given the suspected involvement of S100A7 in skin-related inflammatory responses, and the correlation between S100A7 expression and breast tumor inflammation, we propose that tumor-derived S100A7 may be involved in immune-modulatory processes within the breast tumor microenvironment. How soluble S100A7 affects breast tumor biology is thus a pertinent and unexplored question in breast cancer research.

Relevance of targeting S100A7 in breast cancer
We and others have observed that S100A7 is expressed in ~50% of ductal carcinoma *in situ* (DCIS) lesions, which is a key precursor in the multistage development of breast cancer (6). Perhaps the single most important step in breast cancer progression is the transition of DCIS, a disease limited to the breast duct, to invasive carcinoma, which is capable of local tissue destruction as well as distant spread and establishment of metastases. While S100A7 expression is primarily seen within the *in situ* compartment, it persists in ~20% of invasive tumors, where it is strongly associated with a high grade, estrogen receptor (ER) negative phenotype. Thus, S100A7 expression is associated with the subset of breast lesions that pose the highest risk of progression, and consequently poor patient outcome. Current clinical management of breast cancer features a range of targeted therapeutic strategies for patients with breast tumors which exhibit hormone receptor (ER/PR) and/or growth factor receptor (Her2) positive phenotypes (e.g. tamoxifen and herceptin, respectively). However, for patients with tumors that are ER/PR/Her2 "triple negative" (~20% of invasive lesions), treatment options are limited to relatively nonspecific chemotherapies associated with significant toxicity. Targeted therapies are therefore urgently needed for such breast tumors. Because S100A7 is expressed in ~20% of invasive breast tumors and is specifically associated with ER negative, poor prognosis lesions, S100A7 may be an attractive therapeutic target for breast tumors within this dangerous and challenging subset (3, 16). Pharmacologic inhibition of the S100A7-Jab1 interaction will provide a key means of examining the importance of this signaling mechanism in breast tumor progression. Importantly, small molecules capable of disrupting this interaction may have significant potential as novel targeted breast cancer therapeutics. However, it seems likely that extracellular S100A7 has an additional biological role within the breast tumor microenvironment. As this facet of S100A7 biology is currently uncharacterized, we must first gain a basic knowledge of this field before the clinical use of S100A7-targeted therapies will be justifiable. Thus, understanding the role of extracellular S100A7 is a necessary prerequisite to its potential exploitation as a target for breast cancer therapy.

Body—progress report pertaining to months 1 to 12 in the original SOW

Part A. Structural characterization of the Jab1-S100A7 interaction and testing of small molecule inhibitors of binding.

S100A7 point mutants (Asp56Gly, Leu78Met, and Gln88Lys) were generated and used in yeast-two-hybrid assays to determine their capacity to bind to Jab1. We found that each of the three mutations had a significant impact on S100A7-Jab1 binding, as compared to wild type and triple mutant S100A7 (positive and negative controls, respectively; Figure 1). We had originally assumed that only one (or two at most) of the above mutations were responsible for the loss of biological effects observed for the triple mutant S100A7, and that identifying the important residue would guide the design of further targeted mutations of S100A7. However, since all three mutations appear important for Jab1 binding, we have postponed further mutagenesis experiments pending firmer structural data.
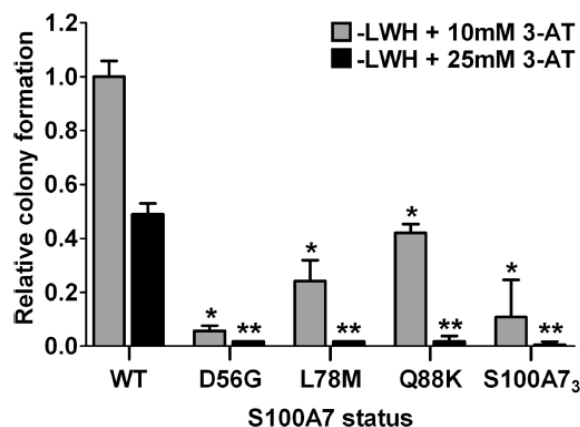

Figure 1. Leu78, Asp56, and Gln88 are each necessary for interaction of S100A7 with its ligand Jab1. Yeast strain Y190 was transformed with Jab1-pGBT9 (bait) and wild-type or mutant S100A7-pACT2.2 (prey) plasmids. Transformants were plated onto leucine/tryptophan/histidine-deficient medium containing 10, 25, or 50 mM 3-aminotriazole (3-AT) to allow increasing strengths of selection. Colonies were counted after 1 week and normalized to growth on leucine/tryptophan-deficient control plates. Data shown represent colony formation relative to clones transformed with wild-type S100A7. No cells grew on media with 50 mM 3-AT, and thus, these data are not shown. Bars represent means of triplicate assays +/- the standard deviation. *$P < 0.001$ and **$P < 0.0001$ relative to wildtype transformants under same selection conditions, based on a Student's t test.

While an effective platform for producing recombinant human S100A7 is in place, our collaborators have been unsuccessful to date in producing recombinant human Jab1 that adopts a native structure in vitro. This has prevented us from obtaining detailed structural and physical data on S100A7-Jab1 binding. However, in order to begin development of a small molecule inhibitor of S100A7-Jab1 binding, we probed S100A7 with a series of fluorescent dyes and determined the crystal structure of S100A7 bound to the napthalene-based molecules 2,6-ANS and 1,8-ANS. These dyes occupied a distinct cleft formed partially by the Leu78 residue that is mutated in the S100A7 triple mutant. We feel that this study may have delineated a useful region

for designing targeted agents for the disruption of S100A7-Jab1 binding. These data, along with the above yeast-two-hybrid results, were published in the journal *Biochemistry* in November 2009:

León R, Murray JI, Cragg G, Farnell B, West NR, Pace TC, Watson PH, Bohne C, Boulanger MJ, Hof F. (2009). Identification and characterization of binding sites on S100A7, a participant in cancer and inflammation pathways. *Biochemistry* **48**: 10591–600.

Work is ongoing to generate small molecules designed from 2,6/1,8-ANS that interact with S100A7 with high affinity and specificity. Lead molecules generated from this approach will be used in bioassays of S100A7-Jab1 interaction. Our assay platforms include the family of MDA-MB-231 cells expressing wild type and triple mutant S100A7 that have been used previously, as well as HEK-293 cells transfected to inducibly express wild type or mutant S100A7 under the control of a Tet-on system, as described in our original proposal.

   In addition to wild type S100A7, we have also generated recombinant S100A7 triple mutant protein. Determination of the triple mutant crystal structure revealed that its overall structure is highly conserved relative to the wild type protein. Structural perturbations are found only in the immediate vicinities of the three mutated residues, the largest of which surrounds the Asp56 site, which was also the most important residue for Jab1 interaction as determined by the yeast-two-hybrid results described above. These results suggest that the loss of biological effects associated with the triple mutation of S100A7 are due to highly specific changes in protein structure, rather than global structural perturbation. These results were published in December 2009 in *Protein Science*:

West NR, Farnell B, Murray JI, Hof F, Watson PH, Boulanger MJ. (2009). Structural and functional characterization of a triple mutant form of S100A7 defective for Jab1 binding. *Protein Sci* **18**: 2615–23.

   Given the unexpected difficulties in producing functional recombinant Jab1, we are currently unable to proceed with the original plan of testing rationally designed small molecules stemming from structural data of the Jab1-S100A7 complex. Although we currently have cell models available with which to test novel drug leads, the current roadblock is in generating such molecules for testing.

Part B. Extracellular roles of S100A7 in the breast tumor microenvironment.

Immunohistochemical staining of our 255 patient cohort of ER– ductal breast carcinomas has proceeded as planned. Tissue microarrays (already stained for S100A7 with accompanying histopathological and clinical data) were stained for the following markers of tumor-infiltrating leukocytes (TIL): CD3, CD4, CD8, CD20, TIA1, FOXP3, CD25, CD68, and myeloperoxidase (MPO). For each marker, the median number of infiltrating cells was used as the cut-point to define high versus low cases. In support of an interaction between S100A7 and the immune system, we observed that cytosolic S100A7 was associated with reduced disease-free survival (DFS) in tumors with high levels of infiltrating CD3^{+} T cells but not in those with low T cell infiltration (Figure 2a). Within the CD3 high subset, S100A7 correlated negatively with

intratumoral levels of CD3^{+} T cells ($P = 0.0079$), as well as cells expressing markers of activated cytotoxic T cells: CD8 ($P = 0.0257$), CD25 ($P = 0.0240$), and TIA1 ($P = 0.0369$). Surprisingly, this is in contrast with the premise that S100A7 is a T cell chemoattractant. Intriguingly, stromal CD25^{+} TIL were a strong prognostic marker in S100A7+ cases, but not those lacking S100A7 expression (Figure 2b). Together, these data suggest that a clinically relevant relationship exists between S100A7 expression and anti-tumor immunity. A manuscript including this data is currently in preparation. Investigation of possible mechanisms to explain these observations (including S100A7-leukocyte chemotaxis experiments) are expected to commence within the next few months.
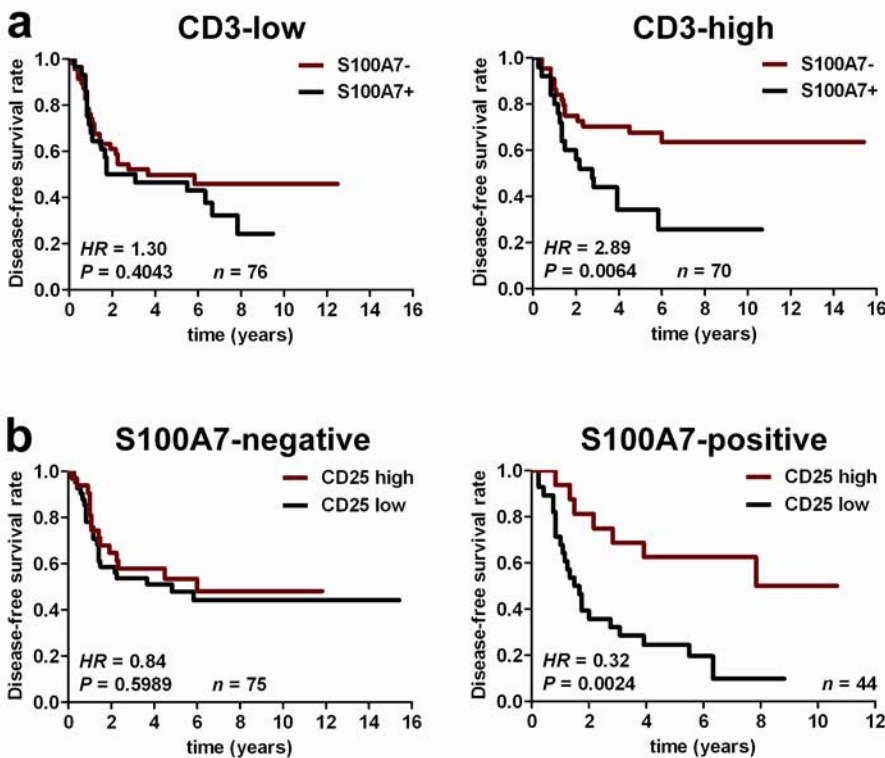

Figure 2. Prognostic relationship between tumoral S100A7 expression and TIL. (**a**) S100A7 expression correlates with reduced DFS in CD3-high but not CD3-low cases. (**b**) CD25^{+} TIL predict improved DFS in S100A7+ but not S100A7- cases. *P*-values determined by log-rank test.

As indicated in the original statement of work, we investigated the effects of inflammatory cytokines on S100A7 expression in breast cancer cells. Key observations from this study include the following:

- Several inflammatory cytokines including oncostatin-M (OSM), interleukin (IL)-6, IL-1β, and TNFα can induce de novo S100A7 expression in MCF7 breast cells, with OSM having the most pronounced effect (Figure 3).
- Induction of S100A7 by OSM and IL-6 is dependent on the PI3K, ERK1/2, and STAT3 signal transduction pathways.

- S100A7 is required for the acquisition of enhanced migratory capacity downstream of OSM and IL-6 stimulation.
- S100A7 expression correlates with that of the OSM receptor (OSMR) *in vivo*, and breast tumors co-expressing S100A7 and OSMR have a poor prognosis (Figure 4).
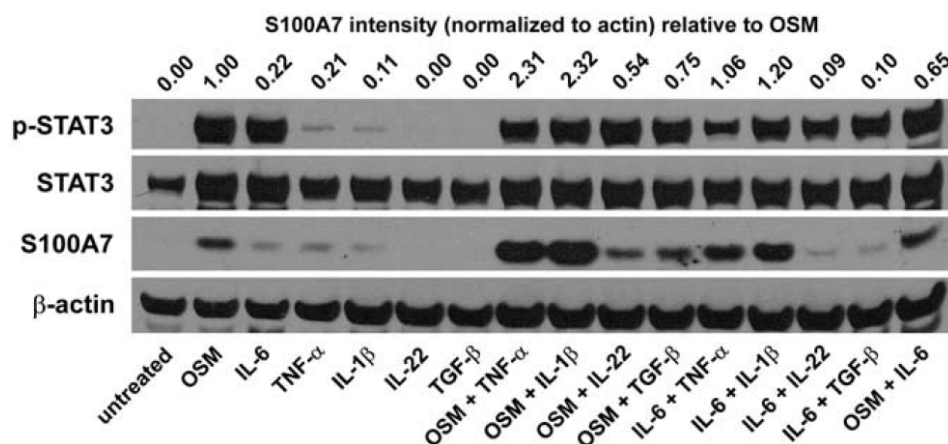

Figure 3. Inflammatory cytokines induce de novo S100A7 expression in MCF7 cells. Cells were stimulated with the indicated cytokines either singly or in combination (all 100 ng/ml) for 24 hours before harvesting for western blot. Band intensities were assessed using ImageJ.
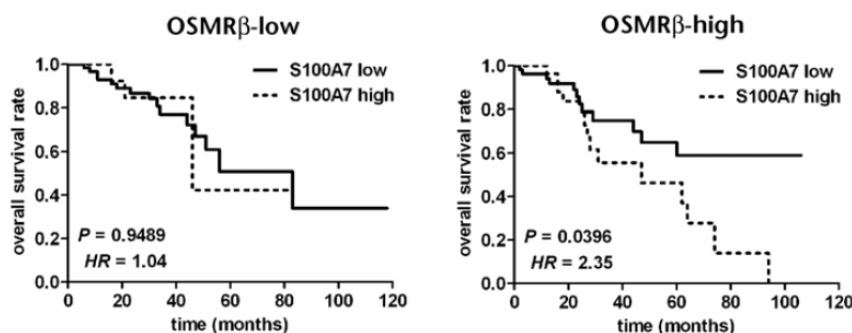

Figure 3. **S100A7 correlates with poor clinical outcome based on OSMRβ expression status**. Publically available and previously published microarray gene expression data were extracted from the University of North Carolina microarray database (Herschkowitz JI *et al*. (2007). Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biol* **8**: R76. (http://genomebiology.com/2007/8/5/R76)). Patients were sorted into OSMRβ high (Log2 expression ratios above median, $n = 85$) and low (below median, $n = 78$) expression groups. High S100A7 expression is defined as a Log2 expression ratio greater than 1. Kaplan- Meier curves were generated using GraphPad Prism 5.0 (*P*-values calculated using the Log-rank test). HR, hazard ratio.

These data were submitted to *Oncogene* and accepted in December 2009 (see Appendix 1, an attached manuscript proof):

West NR, Watson PH. (2010). S100A7 (psoriasin) is induced by the proinflammatory cytokines oncostatin-M and interleukin-6 in human breast cancer. *Oncogene*, in press.

Key research accomplishments

- Generated yeast-two-hybrid data indicating that the S100A7 residues Asp56, Leu88, and Gln88 are each independently necessary for efficient interaction of S100A7 with Jab1.
- Helped determine the crystal structure of the Jab1-binding-deficient S100A7 triple mutant.
- Helped explore a small molecule binding pocket on S100A7.
- Completed IHC staining of breast TMA for TIL markers—led to identification of prognostically relevant relationship between S100A7 expression and TIL density.
- Identified S100A7 as a functionally and clinically relevant inflammation-inducible protein in breast tumor cells.

Reportable outcomes

Abstracts

West NR, Barnes RO, Olson M, Watson PH. Expression of S100A7 in breast cancer is induced by the proinflammatory cytokines OSM and IL-6. American Association for Cancer Research Annual Meeting, April 18-22, 2009, Denver, CO.

Published manuscripts

West NR, Watson PH. (2010). S100A7 (psoriasin) is induced by the proinflammatory cytokines oncostatin-M and interleukin-6 in human breast cancer. *Oncogene*, in press.

West NR, Farnell B, Murray JI, Hof F, Watson PH, Boulanger MJ. (2009). Structural and functional characterization of a triple mutant form of S100A7 defective for Jab1 binding. *Protein Sci* **18**: 2615–23.

León R, Murray JI, Cragg G, Farnell B, West NR, Pace TC, Watson PH, Bohne C, Boulanger MJ, Hof F. (2009). Identification and characterization of binding sites on S100A7, a participant in cancer and inflammation pathways. *Biochemistry* **48**: 10591–600.

Conclusion

In year 1, Tasks A.1-3 were to pursue the structural characterization of the S100A7-Jab1 interaction. We have completed and/or made significant progress in Tasks A.1 and A.3, but unexpected findings in A.1 and roadblocks in the generation of recombinant Jab1 have impeded progress in Tasks A.2 and A.3 at this time. Tasks B.1-3 were to investigate immune cell profiles in tumors and explore the effects of S100A7 and cytokines on breast cells. We have made significant progress on Tasks B.1 and B.3, and are currently initiating task B.2. Data from a clinical cohort of ER– breast cancers suggest that S100A7 and tumor infiltrating leukocytes have a prognostically relevant relationship that warrants further study. Finally, S100A7 can be

induced de novo in breast cancer cells by specific inflammatory cytokines, one of which (OSM) has a clinically relevant association with S100A7 *in vivo*.

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Appendix A—see attached manuscript

npg

ORIGINAL ARTICLE

S100A7 (psoriasin) is induced by the proinflammatory cytokines oncostatin-M and interleukin-6 in human breast cancer

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S100A7 promotes aggressive features in breast cancer, although regulation of its expression is poorly understood. As S100A7 associates with inflammation in skin and breast tissue, we hypothesized that inflammatory cytokines may regulate S100A7 in breast cancer. We therefore examined the effects of several cytokines, among which oncostatin-M (OSM) and the related cytokine, interleukin (IL)-6, showed the most significant effects on S100A7 expression in breast tumor cells *in vitro*. Both cytokines consistently induced S100A7 expression in three cell lines (MCF7, T47D and MDA-MB-468) in a dose- and time-dependent manner. Induction of S100A7 was inhibited by blockade of STAT3, phosphatidylinositol 3 kinase (PI3K) and ERK1/2 signaling and small interference RNA (siRNA)-mediated knockdown of S100A7 eliminated the promigratory effects of OSM treatment. S100A7 mRNA levels in a case-control cohort of breast tumors ($n = 20$) were significantly associated with expression of the OSM receptor β (OSMRβ) chain ($P = 0.0098$). This association was confirmed using publicly available microarray data from an independent breast tumor cohort ($n = 201$, $P = 0.0005$) and a correlation between S100A7 and poor patient survival was observed specifically in cases with high OSMRβ expression ($HR = 2.35$; $P = 0.0396$; $n = 85$). We conclude that inflammatory cytokines can regulate S100A7 expression and that S100A7 may mediate some of their effects in breast cancer.
Oncogene (2010) **0**, 000–000. doi:10.1038/onc.2009.488

Introduction

A growing body of evidence reveals that the host immune system can paradoxically promote or suppress tumor development depending on the disease context and immune players involved. Although adaptive, cell-mediated immune responses can induce tumor regression, many tumors harbor chronically activated infiltrates of innate immune cells and dysfunctional lymphocytes that can support tumor progression (Balkwill *et al.*, 2005; de Visser *et al.*, 2006; Zitvogel *et al.*, 2006). Tumors may use numerous mechanisms to exploit immune activity, including adaptation and responsiveness to immune-derived cytokines. Interleukin (IL)-6, for example, a prominent inflammatory cytokine, was recently shown to promote spheroid formation by breast tumor progenitor cells via Notch signaling (Sansone *et al.*, 2007).

Along with others, we have identified S100A7 (psoriasin) as an inflammation-associated protein relevant to breast tumor progression (Emberley *et al.*, 2004a, b; Krop *et al.*, 2005). S100A7 is over-expressed in ductal breast carcinomas, in which it associates with aggressive, high grade, estrogen receptor alpha (ER)-negative tumors, prominent leukocyte infiltration and poor patient outcome (Al-Haddad *et al.*, 1999; Emberley *et al.*, 2003a, b; 2004a, b), consistent with its stimulatory effects on pro-survival and invasive pathways in breast cells, including PI3K, nuclear factor-κB (NFκB) and AP-1 (Emberley *et al.*, 2005). Although S100A7 can have both pro- and anti-tumorigenic effects *in vitro*, it consistently promotes tumorigenesis *in vivo* based on two breast xenograft mouse models, in which its expression is either upregulated in MDA-MB-231 cells or reduced in MDA-MB-468 cells (Emberley *et al.*, 2003a, b; Krop *et al.*, 2005; Paruchuri *et al.*, 2008). Although current evidence supports a role for S100A7 in promoting breast cancer, the dominant mechanisms remain to be elucidated. Furthermore, the discordance between *in vitro* and *in vivo* assays suggests that more careful consideration of the effects of the extracellular environment on S100A7 regulation and function may be required. S100A7 expression can be induced *in vitro* by several nonspecific stresses such as serum deprivation and high-cell density, suggesting that it may be a stress response gene (Enerbäck *et al.*, 2002). In addition, S100A7 is cooperatively repressed by c-myc and BRCA1 (Kennedy *et al.*, 2005) and is positively regulated by ERβ (Skliris *et al.*, 2007). Nevertheless, there is a lack of information regarding well-defined environmental cues that transduce S100A7-regulatory signals.

Several groups have identified S100A7 as an epidermal response gene to inflammatory cytokines, including oncostatin M (OSM) (Gazel *et al.*, 2006; Liang *et al.*,

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2006). OSM is part of the IL-6 cytokine family, members of which signal through a ubiquitously expressed common receptor chain, gp130, in complex with either the OSM receptor (OSMR)β or IL-6Rα chain. By virtue of the shared activation of gp130, both OSM and IL-6 induce similar signaling cascades, involving STATs, mitogen-activated protein kinases and the phosphatidylinositol 3 kinase (PI3K) pathway (Heinrich et al., 2003).

OSM and IL-6 regulate pleiotropic functions during inflammation. For example, IL-6, along with tumor necrosis factor (TNF)-α and IL-1β, contributes to the classically activated pro-inflammatory macrophage phenotype (Mosser and Edwards, 2008) and OSM may promote neutrophil recruitment to inflamed tissues (Modur et al., 1997). Both cytokines are also implicated in breast cancer progression (Jorcyk et al., 2006; Grivennikov and Karin, 2008). Several cell types may be responsible for producing these cytokines in tumors. However, while IL-6 is often expressed at high levels by malignant cells (Royuela et al., 2004), in addition to fibroblasts and inflammatory cells, lymphocytes and macrophages may be the principal source of OSM in vivo (Liu et al., 1998). As S100A7 associates with inflammation and promotes aggressive features in breast tumors, we set out to examine the possibility that inflammation may regulate and exert pro-tumorigenic effects through S100A7.

Results

Inflammatory cytokines induce S100A7 expression in breast cells
To determine if inflammatory cytokines can regulate S100A7 expression, we treated MCF7 cells for 24 h with 100 ng/ml of IL-6, TNF-α and IL-1β, as well as OSM and IL-22, two inflammatory cytokines known to regulate S100A7 in the epidermis (Boniface et al., 2005; Gazel et al., 2006). We observed a robust induction of S100A7 protein after OSM treatment, and a more modest increase in response to IL-6, TNF-α

and IL-1β (Figure 1). In spite of its role in regulating S100A7 in skin, IL-22 had no apparent effect, nor did TGF-β, generally classified as an immunosuppressive cytokine (Zou, 2005). Synergistic effects on induction of S100A7 were observed when cells were costimulated with combinations of OSM or IL-6 and TNF-α or IL-1β (Figure 1). Our subsequent experiments focused on OSM and IL-6, as OSM was the strongest single inducer of S100A7 and IL-6 is in the same cytokine family.

We found S100A7 to be dose-dependently induced by OSM in MCF7 cells, with a minimum effective concentration of 10–15 ng/ml and a maximally effective concentration of 100 ng/ml (Figure 2a). This effect was also time-dependent at both the protein and mRNA levels (Figures 2b and c). S100A7 mRNA and protein were upregulated by 6 and 24 h post-stimulation, respectively, and peaked at 48–96 h. As expected, stimulation with IL-6 caused a weaker induction of S100A7 at late time-points when compared with OSM (Figure 2d). Induction of S100A7 cytosolic protein by OSM was confirmed by immunofluorescence microscopy (data not shown).

We next assessed OSM and IL-6 effects on the following cell lines: T47D, MDA-MB-231, MDA-MB-468 and ZR75. Stimulation of MDA-MB-231 or ZR75 cells with either cytokine did not affect S100A7 (data not shown). However, both T47D and MDA-MB-468 cells responded to cytokines by upregulating S100A7 expression (Supplementary Figure S1). MDA-MB-468 cells, which possess high endogenous S100A7 expression, showed only a modest response to cytokine treatment. In contrast, T47D cells (S100A7 negative under normal growth conditions) responded by robustly inducing S100A7 on stimulation with either cytokine.

Chronic OSM exposure induces long term, stimulus-independent S100A7 expression
Inflammatory cells are a highly variable component of the in vivo tumor environment and the concentrations of their cytokine products may fluctuate over time (Balkwill et al., 2005; de Visser et al., 2006; Zitvogel et al., 2006). To determine if short-term stimulation with
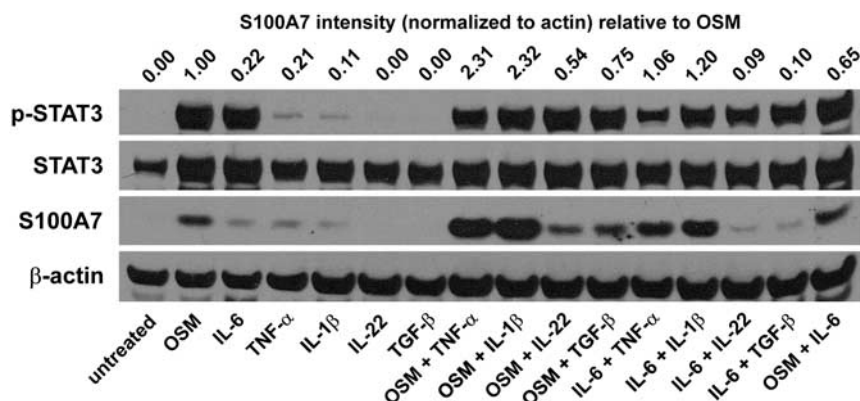

Figure 1 Inflammatory cytokines induce S100A7 in MCF7 cells. Cells were treated with the indicated cytokines (all at 100 ng/ml) singly or in pairs, for 24 h before being harvested for western blot analysis. Actin-normalized S100A7 band densities (relative to the OSM-treated sample) were calculated using ImageJ. Data are representative of three experiments.
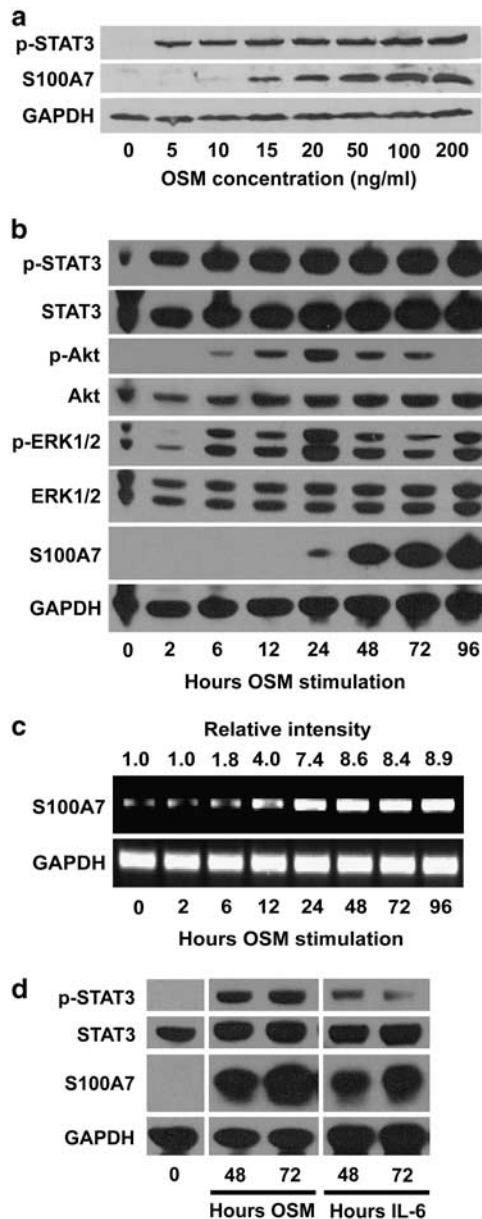
Figure 2 Dose- and time-dependent S100A7 expression after OSM or IL-6 stimulation of MCF7 cells. (**a**) Dose-dependent induction by OSM (western blot). Cells were treated at indicated concentrations for 96 h. (**b**) Time-dependent protein and (**c**) mRNA induction by OSM. Media was spiked with OSM (100 ng/ml) at time = 0 and samples collected for western blot or reverse transcriptase–PCR (RT–PCR) at indicated time points. (**d**) OSM activates STAT3 and induces S100A7 more strongly than IL-6 (western; both cytokines at 100 ng/ml). Data are representative of three experiments.

OSM or IL-6 is sufficient to induce S100A7, we stimulated MCF7 cells for 2 h, after which the cells were washed and cultured in cytokine-free media. In contrast to multi-day treatment, short-term stimulation with OSM was insufficient for full induction of S100A7 expression (Figure 3a), as was short-term IL-6 treatment (Supplementary Figure S2a).

We next examined the long-term persistence of cytokine-induced S100A7 expression. MCF7 cells treated with OSM for 96 h, followed by cytokine removal, showed an approximately 50% reduction in S100A7 mRNA after 72 h in cytokine-free media but maintained high S100A7 protein levels (Figures 3b and c). Similar results were obtained using IL-6 (Supplementary Figure S2b). Although levels of activated STAT3, Akt and ERK1/2 diminished as expected immediately after OSM removal (Figure 3b), these levels recovered soon after, despite the lack of exogenous stimulation. This suggests that long-term stimulation with OSM triggers a mechanism maintaining activation of signaling pathways that may promote persistent S100A7 expression.

OSM induces S100A7 through multiple signaling pathways
OSM or IL-6 stimulation of breast cells triggers several signaling pathways, including those of STAT3, PI3K and ERK1/2 (Heinrich *et al*., 2003). OSM additionally activates the stress-activated mitogen-activated protein kinases p38 and JNK (Heinrich *et al*., 2003). As shown in Figure 2b, STAT3, PI3K and ERK1/2 were effectively engaged over an extended time course by OSM stimulation in our cells.

To identify the pathways necessary for S100A7 expression, we selectively inhibited each of the above kinases during cytokine treatment. Inhibition of either PI3K (using LY294002) or ERK1/2 (using U0126) completely abrogated S100A7 induction by OSM and IL-6 (Figure 4a). Conversely, inhibition of JNK or p38 had no effect (Supplementary Figure S3). Blockade of STAT3 signaling by means of STAT3-specific small interference RNA (siRNA) or over-expression of a dominant-negative STAT3 mutant (Y705F) also inhibited full induction of S100A7 expression (Figures 4b and c). Thus, activation of PI3K, ERK1/2 and STAT3 all appear to be necessary for full S100A7 induction by OSM or IL-6. We (Emberley *et al*., 2005; Wang *et al*., 2008) and others (Paruchuri *et al*., 2008) have previously shown a link between S100A7 and the epidermal growth factor (EGF) pathway. IL-6 and EGF signaling can also be functionally linked (Badache and Hynes, 2001). To begin to explore this connection, we inhibited epidermal growth factor receptor (EGFR) using AG1478 and observed a marked inhibition of S100A7 expression after OSM treatment, accompanied by loss of Akt activation (Supplementary Figure S4). Furthermore, EGF mRNA levels increased during OSM treatment and remained elevated after cytokine removal. Thus, OSM and IL-6 induce S100A7 in MCF7 cells through a complex mechanism involving PI3K, ERK1/2, STAT3, and potentially autocrine EGF signaling.

S100A7 mediates IL-6 and OSM-dependent migration
A hallmark of OSM-stimulated MCF7 or T47D cells is increased migration and invasiveness, accompanied by altered morphology (Zhang *et al*., 2003; Holzer *et al*., 2004; Jorcyk *et al*., 2006). To determine if S100A7 is involved in these previously characterized phenotypic
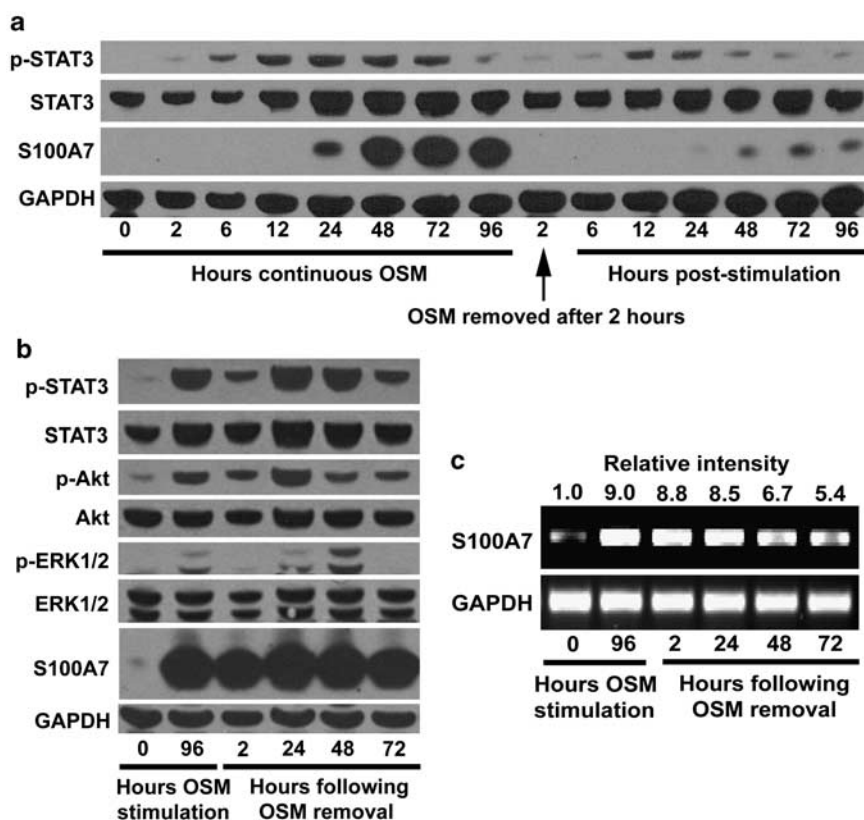
Figure 3 S100A7 induction in MCF7 cells is related to the duration of the OSM stimulus and can persist for several days after removal of cytokine. (**a**) Western analysis of cells stimulated continuously with OSM over 96 h or transiently for 2 h, followed by 96 h in cytokine-free media. (**b**) OSM stimulation for 96 h, followed by 72 h in cytokine-free media (western blot). (**c**) Cells treated as in (**b**), but assessed by reverse transcriptase–PCR (RT–PCR). Data are representative of three experiments.

changes, we used siRNA to inhibit S100A7 expression during cytokine stimulation in MCF7 cells and assessed cell morphology and migratory capacity. Although control transfection with green fluorescent protein-specific siRNA had little effect on OSM-induced morphology change, it was largely prevented by knock-down of S100A7 (Figure 5a). Similarly, siRNA knock-down of S100A7 also abrogated the marked pro-migratory effect of OSM and IL-6 on this cell line, as measured in the under-agar migration assay (Figure 5b, Supplementary Figure S5), showing functional relevance of S100A7 in the context of cytokine stimulation. Knockdown of S100A7 by siRNA was verified by western blot (Figure 5c).

S100A7 is associated with the OSM/IL-6 pathway in vivo
To determine if S100A7 associates with OSM-related genes *in vivo*, we assessed mRNA expression in a cohort of 20 breast tumors. Using semi-quantitative reverse transcriptase–PCR, we examined the mRNA levels of OSM, OSMRβ, IL-6, IL-6Rα, gp130 and LIF (an additional IL-6 family cytokine), normalized to GAPDH (Figure 6a). S100A7 mRNA expression was first reassessed to confirm previously determined S100A7 status based on immunohistochemistry (IHC). We observed 90% concordance between IHC and reverse transcriptase–PCR detection of S100A7, but with two samples previously classified as S100A7 negative by IHC showing weak S100A7 mRNA expression by reverse transcriptase–PCR. These samples were reclassified as S100A7 positive for comparison with mRNA levels of cytokine pathway genes. Only expression of OSMRβ was significantly higher ($P = 0.0098$) in S100A7-positive tumors. (Figure 6b). However, in secondary analysis, in which S100A7-positive status was reassigned to the subgroup of tumors with higher levels of S100A7 mRNA and IHC expression (cases 1–8) versus those with absent or low expression, OSM ($P = 0.041$), IL-6Rα ($P = 0.049$) and OSMRβ ($P = 0.023$) were significantly associated with S100A7-high cases. No significant trends were evident for IL-6, LIF or gp130.

To confirm these observations in a larger independent cohort, we obtained publicly available microarray gene expression data from 201 breast tumors in a previously published data set (Herschkowitz *et al.*, 2007), accessed through the University of North Carolina (UNC) microarray database. Spearman correlation of Log2 normalized expression ratios revealed a strong association between S100A7 and OSMRβ ($P = 0.0005$) that was retained in the ER-negative subset ($P = 0.0013$). Additional correlation data are provided in Supplementary Table S2.
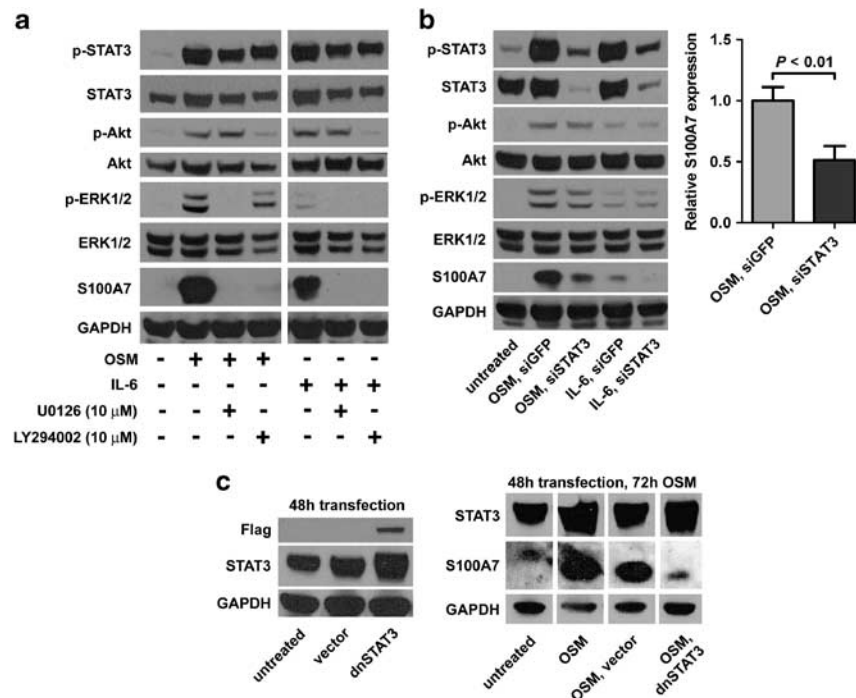
Figure 4 PI3K, STAT3 and ERK1/2 signaling regulate S100A7 in MCF7 cells. (**a**) OSM/IL-6 stimulation for 24 h with inhibitors of ERK1/2 (U0126) or PI3K (LY294002). (**b**) STAT3 inhibition by transfection of STAT3 siRNA 24 h before OSM/IL-6 treatment (left panel). Right panel indicates average (over three experiments, $+/-$ s.d.) S100A7 band densities normalized to GAPDH in OSM-treated cells with or without siSTAT3. (**c**) STAT3 inhibition by transfection with flag-tagged dnSTAT3 mutant (Y705F) 48 h before OSM stimulation. Left panel indicates successful transfection at the time of OSM treatment. Right panel represents cells harvested after 72 h of OSM stimulation. All panels depict western blot data and are representative of at least three experiments.

If S100A7 mediates aggressive features downstream of cytokine stimulation, this may be reflected in clinical patient outcome. Using the UNC data set, we sorted interpretable tumors into those with greater ($n = 85$) or less than ($n = 78$) the median OSMRβ expression level and compared overall survival of patients with high (Log2 ratio >1) and low (Log2 ratio <1) S100A7 expression within each group (Supplementary Figure S6). S100A7 correlated significantly with reduced patient survival within the OSMRβ-high population ($HR = 2.35$, 95% confidence interval 1.04–5.31; $P = 0.0396$), but not the OSMRβ-low population ($HR = 1.04$, 95% confidence interval 0.29–3.74; $P = 0.9489$). Collectively, these data support the notion that OSM and S100A7 are functionally related in breast cancer, and suggest that S100A7 is a poor prognostic factor primarily in the context of tumor inflammation and cytokine signaling.

Discussion

S100A7 is commonly upregulated in breast cancer, most frequently in ductal carcinoma in situ with a high grade, ER-negative phenotype (Emberley et al., 2004a, b). The frequent association of inflammation with this tumor phenotype supports the view that S100A7 may be a chemotactic factor for inflammatory cells (Jinquan et al., 1996; Wolf et al., 2008), although it is also

possible that inflammation may precede and stimulate S100A7. We have shown here that two IL-6 family cytokines, OSM and IL-6, regulate S100A7 in breast cell lines via (in MCF7 cells) PI3K, ERK1/2 and STAT3 signaling. Furthermore, we have shown that S100A7 can mediate the effects of OSM on cell morphology and migration. In addition, we have confirmed that a relationship between the OSM pathway and S100A7 exists in breast tumors in vivo that has relevance to prognosis. It is noteworthy that although we identified four cytokines capable of inducing S100A7 expression in vitro (OSM, IL-6, TNF-α and IL-1β; Figure 1), only the OSM pathway correlates with S100A7 levels in vivo (Figure 6; Supplementary Table S2).

OSM and IL-6 are produced during inflammatory responses by activated T lymphocytes and macrophages (Naka et al., 2002; Tanaka and Miyajima, 2003). Both IL-6 and OSM exert a variety of effects on breast cancer cells and those of other tumor types. Numerous investigators have examined the role of IL-6 in breast cancer and have shown it to exert both pro- and anti-tumor effects in a context-dependent manner (Knüpfer and Preiß, 2007). The role of OSM in cancer has been less well studied. As OSM is cytostatic to breast tumor cells in vitro, it was initially thought to have potential therapeutic value. Subsequent studies, however, have revealed that this potential may be limited by the ability of OSM to enhance migration and invasiveness (Zhang et al., 2003; Holzer et al., 2004; Jorcyk et al., 2006). The effects of OSM and IL-6 may contribute to the common
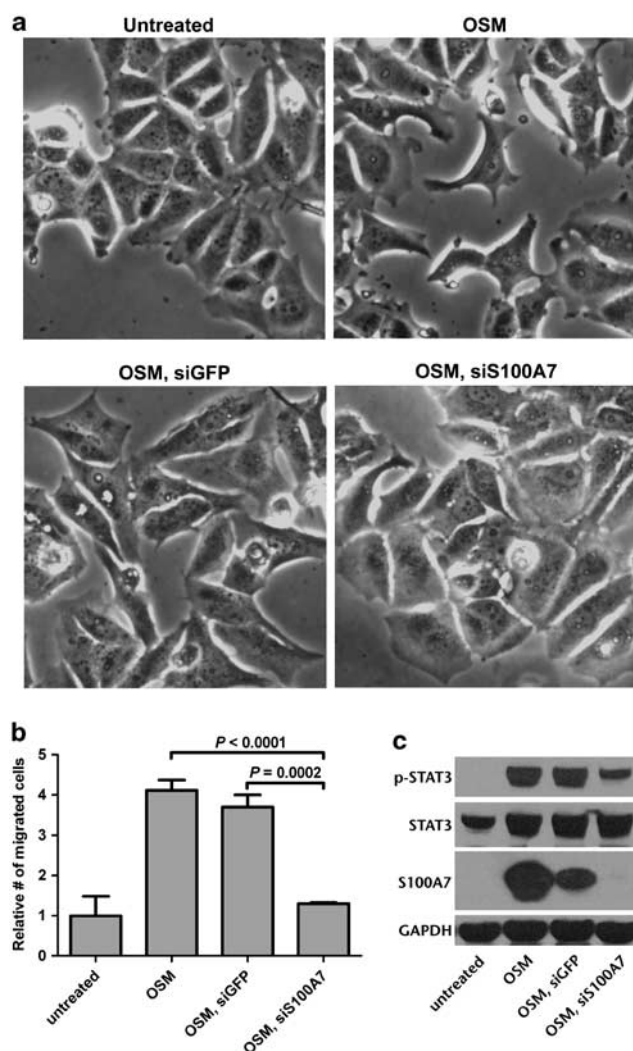
Figure 5 S100A7 mediates OSM-induced migration in MCF7 cells. (**a**) OSM induces loss of adhesion, cell spreading and formation of migratory structures. Transfection with 100 nM S100A7 siRNA, but not green fluorescent protein (GFP) siRNA, 24 h before treatment prevents this phenotype. Original magnification 400 ×. (**b**) S100A7 knockdown prevents migration in OSM-treated cells, as assessed by the under-agar assay (see Materials and methods). Bars represent mean (+/− s.d., $n=3$) numbers of migrated cells normalized to the mean of untreated controls, compared using Student's t-test. The data shown are representative of six experiments. (**c**) Western blot verification of S100A7 knockdown by siRNA.

Figure 6 Association of S100A7 with OSM/IL-6 related genes *in vivo*. (**a**) Twenty frozen breast tumor samples were chosen based on IHC classification of S100A7 expression. Reverse transcriptase–PCR (RT–PCR) revealed S100A7 expression in 12 of 20 samples. PCR products of indicated targets from a representative experiment are shown. (**b**) Comparison of gene expression in samples 1–12 (S100A7+) versus negative controls (13–20). Levels of indicated transcripts were assessed by semi-quantitative RT–PCR. Data points represent *GAPDH*-normalized means of three replicate experiments (bars = medians) compared using Mann–Whitney T-test.

observation that chronic inflammation can promote tumorigenesis (Balkwill *et al.*, 2005; de Visser *et al.*, 2006; Zitvogel *et al.*, 2006). Indeed, we have shown here that OSM may mediate aggressive features via S100A7 expression, and that the OSM/S100A7 axis correlates with reduced patient survival.

Although S100A7 can be highly expressed in breast tumors and is implicated as a gene induced by several forms of cell stress (Di Nuzzo *et al.*, 2000; Enerbäck *et al.*, 2002), specific mechanisms regulating S100A7 in breast cells have remained elusive. Nevertheless, in non-neoplastic skin pathologies, several investigators have
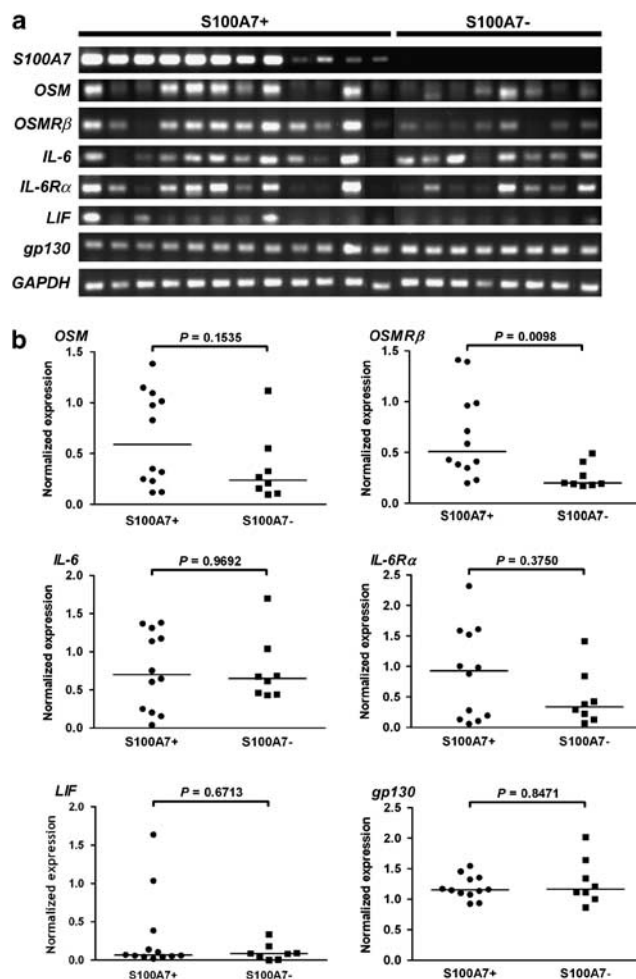
reported S100A7 induction in response to inflammatory cytokines, including OSM, IL-1, IL-17, IL-20 and IL-22 (Boniface *et al.*, 2005; Gazel *et al*, 2006; Liang *et al.*, 2006; Wolk *et al.*, 2006; Bando *et al.*, 2007; Boniface *et al.*, 2007; Sa *et al.*, 2007). Each of these cytokines has the capacity to signal, either directly or indirectly, through STAT3, ERK1/2 and PI3K (Heinrich *et al.*, 2003; Pestka *et al.*, 2004; Gaffen, 2008; Perrier *et al.*, 2008). We have shown previously that S100A7 in breast tumors correlates with Akt activation (Emberley *et al.*, 2005) and now show that OSM and IL-6 can induce S100A7 via PI3K, ERK1/2 or STAT3. We speculate that this may reflect convergence of all three pathways on the same regulator of S100A7. One possible candidate may be a member of the C/CAAT enhancer binding protein (C/EBP) transcription factors. C/EBPβ

and C/EBPδ are both expressed downstream of IL-6 (Conze *et al.*, 2001). Furthermore, the S100A7 promoter contains multiple C/EBPβ recognition sites and S100A7 correlates strongly with C/EBPβ in the UNC microarray data set (Supplementary Table S2). In sum, current data suggest that OSM/IL-6 can induce S100A7 through a complex multi-tiered signaling network, a clearer understanding of which will be an important goal for future study.

In support of the premise that chronic inflammation exacerbates tumor progression, we found that short-term cytokine stimulation of MCF7 cells was insufficient to induce S100A7, suggesting that sustained inflammatory conditions may be required. Although STAT3, ERK1/2 and PI3K mediate S100A7 expression, they may not do so directly, as their activation precedes S100A7 mRNA induction by several hours. As a direct target of these pathways should become rapidly activated, this implies that an additional event(s) takes place. This may be related to signals emanating from changes in adhesion or the cytoskeleton, but further exploration of this possibility will be confounded by the observation that S100A7 itself can mediate these changes.

Alternatively, the delay in S100A7 expression may reflect the time required to achieve a critical threshold of signal strength. Indeed, the levels of activated STAT3, ERK1/2 and Akt increase steadily during the first 24 h of OSM treatment, perhaps as a result of positive feedback (Daur *et al.*, 2005). Furthermore, as a target gene of NFκB, IL-6 expression can be triggered by Akt and maintained by subsequent autocrine stimulation (Yang *et al.*, 2007).

Our data also suggest that high-level induction of S100A7 by OSM may require EGF autocrine signaling. Although our data are preliminary this is intriguing, given previous observations that S100A7 itself may promote EGF expression and is involved in EGFR signaling (Emberley *et al.*, 2005; Paruchuri *et al.*, 2008; Wang *et al.*, 2008). We have confirmed by analysis of expression levels in an independent breast tumor data set (UNC) that S100A7 correlates with EGFR expression in a large unselected cohort and within the ER-negative subset, supporting the existence of functional cooperativity between S100A7 and EGFR (Supplementary Table S2).

Once induced by OSM, S100A7 levels appear to remain elevated after cytokine withdrawal. This suggests that, once expressed at high levels *in vivo*, S100A7 expression may persist despite downstream fluctuations in cytokine concentration. From a practical standpoint, this phenomenon would complicate research efforts directed at correlating S100A7 with specific inflammatory mediators *in vivo*. Stability of the S100A7 mRNA and protein are unknown but may be a factor in this persistence. The closely related S100A8 gene shows an mRNA half life of ∼8 h that can be increased up to 20 h by glucocorticoid treatment (Hsu *et al.*, 2005). Similarly, S100A4 mRNA and protein half life has been shown to be ∼8 h and ∼85 h, respectively (Rivard *et al.*, 2007). However, several other possible mechan-

isms may underlie this observation. First, autocrine production of IL-6 may continuously drive signals that sustain S100A7 expression. Second, S100A7 itself may establish an autocrine loop, as it is secreted and a putative ligand for the receptor for advanced glycation end-products, a promiscuous receptor that signals through NFκB and mitogen-activated protein kinases (Enerbäck *et al.*, 2002; Wolf *et al.*, 2008). Autocrine EGF signaling constitutes another potential mechanism, but is not supported by preliminary evidence (data not shown). Another explanation is based on the role of S100A7 in OSM-induced migration. In MCF7 and T47D cells, fibronectin is upregulated by OSM (Zhang *et al.*, 2003) and, in multiple myeloma, can activate STAT3 in cooperation with IL-6 via β1 integrin signaling (Shain *et al.*, 2009). If S100A7 mediates OSM-induced migration in MCF7 cells, it may also regulate STAT3 through integrin signaling. In turn, sustained STAT3 activity could maintain S100A7, perpetuating a loop of pro-migratory signals. The potential S100A7-regulatory mechanisms described above are summarized in Supplementary Figure S8.

S100A7 is strongly associated with ER-negative status in breast cancer. On the basis of our studies of invasive breast cancer, S100A7 is expressed in approximately 50% of ER-negative versus 20% of ER-positive tumors (Al-Haddad *et al.*, 1999; Emberley *et al.*, 2003a, b, 2004a, b). Similar ratios were observed in ductal carcinoma *in situ*, in which 80% of ER-negative and 30% of ER-positive pure ductal carcinoma *in situ* lesions were S100A7 positive (Emberley *et al.*, 2004a, b). We currently show that OSM/IL-6 can induce S100A7 in ER-positive MCF7 and T47D cells, although the OSM/IL-6 pathway is associated *in vivo* with S100A7-positive tumors that are predominantly ER negative. The reason for the association of S100A7 with ER-negative status *in vivo* is unknown. However, it may be relevant that OSM was shown to dramatically reduce ER expression in MCF7 cells (Grant *et al.*, 2002), an observation that we have verified (Supplementary Figure S7). Furthermore, IL-6 correlates with ER-negative status and poor prognosis in breast cancer patients (Knüpfer and Preiß, 2007). Therefore, it is possible that OSM/IL-6 signaling may downregulate ER and drive ER-positive breast tumors toward an ER-negative phenotype while concurrently upregulating S100A7, providing a rationale for the link between S100A7 and ER-negative tumor status.

It was recently reported that treatment of breast cells with interferon-γ, a signature cytokine of cell-mediated (Th1) immune responses, suppressed endogenous S100A7 expression (Petersson *et al.*, 2007). OSM and IL-6 differ from interferon-γ in that they function more generally as pleiotropic inflammatory signals. Thus, while inflammatory cytokines may positively regulate S100A7, Th1 cytokines could have an opposing effect. This notion is supported by our analysis of microarray data, which reveals a positive correlation between S100A7 and OSMRβ within ER-negative tumors, in contrast to a negative correlation with interferon-γ (Supplementary Table S2).

Although effective anti-tumor adaptive immune responses can be associated with improved clinical outcome, festering intratumoral inflammation is thought to promote malignancy. Indeed, the ability of tumor cells to exploit immune responses for their own benefit by diverse means is increasingly documented (Balkwill *et al.*, 2005; de Visser *et al.*, 2006; Zitvogel *et al.*, 2006). Expression of S100A7 may represent a novel strategy for malignant cells to translate potentially damaging immune responses into beneficial stimuli. It will be important to achieve a broader understanding of the specific immune parameters regulating S100A7 in the breast, which may reveal additional strategies for targeting S100A7 therapeutically. Furthermore, such knowledge could be relevant in the development of cancer immunotherapies, for which S100A7 expression may be a confounding and potentially damaging side effect of treatment.

In conclusion, S100A7 is influenced by the inflammatory cytokines OSM and IL-6 in breast cancer and may regulate their pro-invasive effects. Together with previous data implicating S100A7 in tumor cell survival, these data further support a role for S100A7 in tumor progression. Given the prominence of S100A7 expression in preinvasive ductal carcinoma *in situ*, this also supports the concept that inflammation can influence the earliest stages of breast cancer development through specific modulation of breast cancer gene expression. Further investigation of specific inflammatory conditions regulating S100A7 in breast cancer is warranted, and may reveal novel strategies to target S100A7 therapeutically.

Materials and methods

Cell culture, transfection and cytokine stimulation
Human breast carcinoma cell lines MCF7, T47D, ZR75, MDA-MB-231 and MDA-MB-468 (obtained originally from ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium with 5% fetal bovine serum under standard conditions. Characteristic features of cultured lines (morphology, doubling time, and so on) are continually monitored for detection of potential cross-contamination by light microscopy. For stimulation assays, viable cells were seeded into 12-well plates (Corning, Lowell, MA, USA) at 100 000 cells per well (40 000 for long-term withdrawal assays). Human OSM, IL-6, TNF-α, IL-1β, IL-22 and TGF-β (Peprotech, Rocky Hill, NJ, USA) were stored as 10 μg/ml stocks in culture media. For withdrawal assays, cytokine-spiked media was aspirated, cells were washed twice with sterile phosphate-buffered saline, and further cultured in cytokine-free media. Inhibitors to MEKK1/2 (U0126; Cell Signaling, Danvers, MA, USA), PI3K (LY294002), p38 mitogen-activated protein kinase (SB203580), EGFR (AG1478) or JNK (JNK inhibitor VIII; Calbiochem, San Diego, CA, USA) were added (all 10 μM) to cultures 30 min before cytokine stimulation. Transient transfection of dominant negative, flag-tagged STAT3 (Y705F; in pRc/CMV; Addgene plasmid 8709 (Wen and Darnell, 1997)) or empty vector DNA was performed using Lipofectamine (Invitrogen, Burlington, ON, Canada) 2 days before cytokine stimulation. Transfection of STAT3 or S100A7-specific ON-TARGET plus siRNA (Dharmacon, Lafayette, CO, USA) or green fluores-cent protein-specific siRNA (Qiagen, Mississauga, ON, Canada) was conducted using Dharmafect-4 reagent (Dharmacon). Light micrographs were captured using a QICAM camera (QIMAGING, Surrey, BC, Canada) with an Axiovert 40 CFL light microscope (Zeiss, Toronto, ON, Canada).

Western blot
Cells were prepared for western blotting as described previously (Al-Haddad *et al.*, 1999). Protein concentrations were estimated by absorbance at 280 nm using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Primary antibodies were S100A7 (1:1000; Abcam, Cambridge, MA, USA), GAPDH (1:3000; Stem Cell Technologies, Vancouver, BC, Canada), phospho-STAT3 (Tyr[705]; 1:1000), STAT3 (1:1000), phospho-Akt (Ser[473]; 1:500), Akt (1:1000), phospho-ERK1/2 (Thr[202]/Tyr[204]; 1:500), ERK1/2 (1:1000) and Flag (1:500; Cell Signaling). Secondary antibodies were horseradish peroxidase-conjugated bovine anti-rabbit and goat anti-mouse IgG (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Reverse transcriptase–PCR
Breast tumor samples from primary invasive ductal carcinomas were obtained from the Manitoba Breast Tumor Bank (Winnipeg, MB, Canada), which operates with approval from the research ethics board of the Faculty of Medicine, University of Manitoba. Tissues are accrued to the bank and frozen at –70 °C. A portion of the frozen tissue from each case is processed to create matched formalin-fixed paraffin-embedded and frozen tissue blocks. We constructed a case–control cohort by selecting ten S100A7-positive breast tumors from a larger series of tumors that had been previously assessed by IHC performed on the paraffin blocks (Emberley *et al.*, 2003a, b), and from which frozen tissue blocks were still available, and randomly selected 10 additional S100A7 IHC-negative tumors as controls.

Total RNA was extracted from frozen tissue and cultured cells using the Qiagen RNEasy Mini kit. One μg of RNA per sample was reverse-transcribed using MMLV reverse transcriptase as per manufacturer instructions (Invitrogen). Primer sequences and cycling conditions for PCR are provided in Supplementary Table S1. PCR products were electrophoresed, stained with ethidium bromide and imaged using a FluorChem 5500 (Alpha Innotech, San Leandro, CA, USA). Band intensities were assessed using ImageJ (NIH, Bethesda, MD, USA). Three independent assays were performed per target, per experiment.

Under-agar migration assay
Migration assays were conducted using six-well plates (Corning) containing semi-solid base-layers of Dulbecco's modified Eagle's medium, 5% serum and 0.5% sterile agar (2.5 ml per well). One-cm round plugs were removed from base layers using a bore, producing cylindrical wells within the agar and exposing plate plastic. Equal numbers of viable cells were plated into the agar-wells and cultured for 24 h. Cells that migrated away from well edges (excluding those still in contact with well edges) were counted in three high-power fields (400 ×) per well and averaged. Each experiment was performed in triplicate.

Statistical analyses
Migration assay results were analyzed using Student's *t*-test. Tissue mRNA levels were compared using Mann-Whitney *T*-test. *P*-values <0.05 were considered statistically significant. All operations were performed using Prism 5.0 (GraphPad, La Jolla, CA, USA).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Title:
Exploring and Exploiting the Protein S100A7 as a New Target for Breast Cancer Therapy

Principal Investigator:
Nathan West, B.Sc

Contracting Organization:
British Columbia Cancer Agency, 555 12 AVEWSTE 400, Vancouver, BC, V5Z 1L3

Date:
January 2010

Request for revision of original Statement of Work

Prepared for:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

Original statement of work: "Exploring and Exploiting the Protein S100A7 as a New Target for Breast Cancer Therapy."

Months	Tasks
	Part A. Structural characterization of the Jab1-S100A7 interaction and testing of small molecule inhibitors of binding.
1-4	1. Generation and characterization of S100A7 point mutants at the following sites: Asp56, Leu78, and Gln90 (residues implicated in Jab1-binding by data from previous studies; mutants will be tested for Jab1 binding by yeast-2-hybrid assay, co-immunoprecipitation, and co-localization immunofluorescence in breast cells);
5-12	2. Mutation of other S100A7 residues implicated in Jab1 binding (identified via computational modeling and homology to ligand binding residues in other S100 proteins); characterization of mutant S100A7-Jab1 interactions, as above; ***Report on structural basis of S100A7-Jab1 binding*
5-12	3. *In vitro* testing of first generation small molecule inhibitors in cell models (inhibitors designed based on preliminary data; e.g. computational protein modeling of S100A7); valid inhibitors will antagonize the following processes in S100A7-expressing breast cells: nuclear accumulation of Jab1, enhanced AP-1 activity, and p27^{KIP1} degradation;
12-36	4. *In vitro* testing of 2nd and 3rd generation inhibitors, designed based on data from mutagenesis studies (and potentially crystallography data from our collaborators); ***Report on design, activity, and biological effects of S100A7-Jab1 inhibitors*
24-36	5. *In vivo* testing of inhibitors validated *in vitro* using nude mouse xenograft preclinical models; successful inhibitors will antagonize growth of S1007-expressing tumors and improve animal survival outcomes. ***Report on in vivo efficacy and therapeutic potential of S100A7-Jab1 inhibitors*
	Outcomes and deliverables: Determining the structural basis of S100A7-Jab1 interaction will provide insight to this important aspect of S100A7/Jab biology. Inhibitors which prevent Jab1-S100A7 association and activation of downstream pathways *in vitro* will be useful in addressing future questions regarding the S100A7-Jab1 pathway. Furthermore, inhibitors which show antitumor efficacy *in vivo* will be candidates for human clinical trials in the treatment of S100A7(+) breast tumors.
	Part B. Extracellular roles of S100A7 in the breast tumor microenvironment.
1-4	1. IHC staining and analysis of breast tumor TMA to identify infiltrating leukocyte subsets which correlate with S100A7 expression (if quality of existing TMA is poor, more time will be required (~2 months) to build and assemble a new cohort of cases for TMA, from tissues banked at the Manitoba and BCCA tumor tissue repositories); ***Report on clinical-pathological data regarding S100A7 and inflammation in vivo*
5-16	2. Investigation of chemotactic responses of leukocyte subsets identified in TMA to soluble S100A7 using transwell and transendothelial migration assays;
5-24	3. Treatment of breast cell lines with cytokines (those known to be produced by leukocytes identified in TMA) to explore effects of leukocytes on S100A7 expression, as well as the relevant signal transduction pathways affecting S100A7;

12-24	4. Investigation of activation/effector responses of leukocytes identified in TMA to soluble S100A7 using flow cytometry (to detect markers of activation following treatment of human peripheral blood leukocytes with S100A7); **Report on functional relationship between S100A7 and inflammation in the breast*
24-36	5. Based on data from the above tasks, planning and execution of further experiments to delve deeper into the extracellular roles of S100A7 in the tumor microenvironment. Outcomes and deliverables: S100A7 is known to affect immune activity in skin and is associated with inflammation in breast cancer. This study will address the potential immunomodulatory role of S100A7 in the breast tumor microenvironment. Specifically, this study will determine the following: (i) which leukocyte subtypes are specifically associated with S100A7 expression in the breast; (ii) the underlying mechanism of this association (i.e., recruitment of leukocytes due to S100A7 expression, S100A7 expression due to signals from leukocytes, or both), and (iii) the biological responses of specific leukocyte subtypes to soluble S100A7. Furthermore, as this field is currently unexplored, this study will provide a basis for a potentially broad range of future investigations, and will support the potential exploitation of S100A7 as a target for breast cancer therapy. **Training plan/presentation milestones**.
1-12	Presentation of research at the BC Cancer Agency and AACR conferences;
12-16	First round of publications (one each for Parts A and B);
13-24	Presentation of research at BCCA, AACR, and DOD BCRP conferences;
24-30	Second round of publications (one each for Parts A and B);
25-36	Presentation of research at BCCA and AACR conferences;
30-36	Publication of *in vivo* inhibitor data (Part A).

Tasks to be removed:
As described in our progress report for 2009, we believe we have made excellent early progress towards completion of Part A tasks but have nevertheless recently encountered significant roadblocks in completing the objectives for Part A (shaded in grey). Specifically, we have been unable to produce functional recombinant Jab1, which has prevented elucidation of the S100A7-Jab1 costructure. This in turn has delayed development of drug leads. As these tasks are primarily the responsibility of our collaborators, we have little direct control over these issues, and our future contribution to this project is uncertain pending their resolution. Although this collaboration will remain active, we are requesting that these tasks be dropped for the purposes of this grant.

Replacement of Part A:
Significant progress has been made on the tasks outlined in Part B. An intriguing observation emerged from the investigation of cytokine-regulation of S100A7 (see Appendix A of the annual report). We noted that oncostatin-M (OSM) stimulation of MCF7 and T47D cells resulted in a significant decrease in estrogen receptor alpha (ER) mRNA expression, in conjunction with gain of S100A7 expression and migratory capacity. This suggests that inflammatory cells can potentially control ER expression in breast cancer via production of specific cytokines such as OSM. Interestingly, IL-6 (an OSM family member) can induce an epithelial-mesenchymal transition (EMT) in breast cells, a phenotype commonly observed

in ER– breast tumors. ER status is an important phenotypic and prognostic marker in breast cancer and is vital in predicting response to endocrine therapy. Despite our extensive knowledge of the role of ER in breast cancer, the mechanisms by which breast tumors lose ER expression are poorly understood. We hypothesize that OSM derived from intra-tumoral inflammation suppresses ER and promotes invasion (or an EMT-like phenotype) in breast tumor cells and that S100A7 is linked to the mechanism of action and promotes this type of inflammatory response. The specific aims pertaining to this hypothesis that we would like to pursue in replacement of Part A (for years 2-3 of funding) are as follows:

AIM 1. What role does S100A7 play in the mechanism of OSM suppression of ER and induction of EMT-like properties in breast tumor cells?
We have shown that OSM upregulates S100A7, downregulates ER, and exerts effects on a single aspect of an EMT-like phenotype (migration) that requires S100A7. It is possible that the effects of OSM on these parameters occur through independent signaling pathways diverging from the established signaling intermediates of OSMR (STAT3, PI3K/AKT, or ERK). It is also plausible that S100A7 plays a role as a necessary factor in both ER suppression and EMT induction. Finally it is possible that ER suppression is a consequence of EMT. We will delineate the pathways by manipulating signaling and gene expression in cell lines in-vitro and in-vivo, initially focusing on ER and EMT issues separately.

AIM 2. What is the in-vivo evidence that S100A7 mediates the actions of the OSM/OSMR pathway in breast tumor progression? We will address this through three subaims as follows;
A). Determination of the cellular origins of OSM/OSMR expression in breast tumors.
B) Determination of the association of OSM/OSMR/S100A7 with molecular subtypes of breast cancer.
C) Determination of the significance of OSM/OSMR/S100A7 in relation to endocrine therapy.

For detailed explanation of the methodology and experimental procedures to be used in the execution of these aims, please see the attached proposal (Appendix A, Aims 1 and 2).

We feel that the work proposed in our revised aims will significantly contribute to our understanding of an important biological and clinical aspect of breast cancer biology, namely, the regulation of ER expression. Furthermore, the proposed work is highly relevant to the aims described in Part B of the original statement of work, as S100A7 may be part of a regulatory loop involving its induction by inflammation, followed by S100A7-regulated effects on both breast cell and leukocyte biology.

Revised Statement of Work Timeline:

Months	Tasks
	Part A. Extracellular roles of S100A7 in the breast tumor microenvironment.
1-4	1. IHC staining and analysis of breast tumor TMA to identify infiltrating leukocyte subsets which correlate with S100A7 expression (if quality of existing TMA is poor, more time will be required (~2 months) to build and assemble a new cohort of cases for TMA, from tissues banked at the Manitoba and BCCA tumor tissue repositories);
5-16	2. Investigation of chemotactic responses of leukocyte subsets identified in TMA to soluble S100A7 using transwell and transendothelial migration assays;
5-24	3. Treatment of breast cell lines with cytokines (those known to be produced by leukocytes identified in TMA) to explore effects of leukocytes on S100A7 expression, as well as the relevant signal transduction pathways affecting S100A7;
12-24	4. Investigation of activation/effector responses of leukocytes identified in TMA to soluble S100A7 using flow cytometry (to detect markers of activation following treatment of human peripheral blood leukocytes with S100A7);
24-36	5. Based on data from the above tasks, planning and execution of further experiments to delve deeper into the extracellular roles of S100A7 in the tumor microenvironment.
	Part B. S100A7, inflammation, and ER regulation.
12-24	1. Elucidation of signaling/regulatory mechanisms controlling S100A7, ER, and EMT gene expression downstream of inflammatory cytokine signaling. Also, delineation of the functional relevance of S100A7 in this process.
12-36	2. Determination of cellular origins of OSM/OSMR in breast cancer.
12-36	3. Determination of the association of OSM/OSMR/S100A7 with molecular subtypes of breast cancer.
12-36	4. Determination of the significance of OSM/OSMR/S100A7 in relation to endocrine therapy.
	Outcomes and deliverables: S100A7 is known to affect immune activity in skin and is associated with inflammation in breast cancer. This study will address the potential immunomodulatory role of S100A7 in the breast tumor microenvironment. Specifically, this study will determine the following: (i) which leukocyte subtypes are specifically associated with S100A7 expression in the breast; (ii) the underlying mechanism of this association (i.e., recruitment of leukocytes due to S100A7 expression, S100A7 expression due to signals from leukocytes, or both), and (iii) the biological responses of specific leukocyte subtypes to soluble S100A7. In addition, we will examine the biological and clinical relevance of S100A7 in the context of ER regulation and EMT-like phenotypes downstream of inflammatory cytokine signaling. Furthermore, as this field is currently unexplored, this study will provide a basis for a potentially broad range of future investigations, and will support the potential exploitation of S100A7 as a target for breast cancer therapy.

Signed by the Principal Investigator on January 26, 2010.

Nathan R. West, B.Sc.
Graduate student, University of Victoria & BC Cancer Agency

Appendix A on following page.

APPENDIX A

The role of inflammation and the OSM-S100A7 Pathway in breast cancer progression

A. RATIONALE
The recent recognition of the role of inflammation in promoting the progression of breast cancer to more invasive, metastatic, and treatment unresponsive status, offers new potential avenues for therapy. These avenues may be particularly important in breast cancer subtypes such as 'triple negative' breast tumors where options for targeted therapies are currently lacking. However the interaction between inflammation and tumor is complex and specific pathways for therapeutic targeting need to be resolved. We believe that the inflammation derived cytokine Oncostatin M (OSM) is an important factor in promoting invasion and resistance to endocrine therapy. We also believe that S100A7 is a key component and biomarker of OSM action in breast tumor cells. This study will explore these concepts in cell line models and human tumors and determine potential mechanisms that might help to identify novel therapeutic strategies for ER negative breast cancer.

BACKGROUND – on relevant genes, pathways and therapies in breast cancer,
B.1. Breast Cancer
Recent therapeutic advances in breast cancer have been most impressive for targeted therapies, such as those targeting the estrogen receptor (ERα) and the epidermal growth factor-receptor 2 (Her2), specifically benefiting the subsets of patients defined by these biomarkers. Other subsets of tumors remain difficult to treat. The ERα negative phenotype, which includes the so-called 'triple negative' category (ERα-/PR-/Her2-), has therefore dominated clinical and biological consideration of breast cancer for many years.

B.2. Estrogen receptor (ER) and Breast Cancer Progression
Estrogen receptor (ER) is a central factor in breast cell biology and growth. ERα (henceforth referred to as ER) is a transcription factor that mediates estrogen signaling. ER is both a target for endocrine therapy and a biomarker for molecular breast cancer phenotypes (ie the "intrinsic" subtypes luminal A, luminal B, HER2-enriched, and basal-like).[1] At clinical presentation ~30% of primary tumors are ER- while ~70% are ER+ (and the former are mostly intrinsically resistant while the latter are mostly responsive to endocrine therapy).[2] One view is that resistance stems from intrinsic epigenetic repression of ER in early tumorigenesis (eg through promoter hypermethylation)[3] leading to ER- tumors.[4] Another view is that resistance can develop as a result of evolution of ER+ to ER- tumors or from acquired mechanisms that lie downstream of ER in ER+ tumors (eg altered coregulators and signal transduction pathways).[5] The recent major focus to understand acquired resistance has been on mechanisms that lie downstream of ER. However we now know that ER is often reduced and can be lost with progression in 25-30% of ER+ tumors.[6-8] Also, detailed expression array analysis has identified subsets of ER- tumors (~25%) that share profiles with subsets of ER+ tumors (eg 'ER response genes' and 'MAPK' signatures.[9,10] Furthermore several mechanisms whereby downregulation of ER might occur during tumor progression have emerged. These include acquired intracellular alterations in kinase signaling pathways[10,11] and extracellular factors such as hypoxia (our own work[12]). Overall, these data support the notion that at least some ERlow+ and ER- tumors may represent

'ER suppressed' tumors, with reversible depression of ERα caused by intra-tumoral or environmental factors.

B.3. Inflammation

A growing body of evidence has revealed that the host inflammatory and immune response can influence tumor progression. It is also now clear that the immune cell compartment can directly influence tumor cell differentiation[13] and that the tumor cell can directly sculpt this network through cytokine production that can influence the composition and activities of the inflammatory and immune cell compartment. In general terms, acute inflammation and immune responses drive tumor rejection and are dominated by CD4 T-cells and M1 macrophages producing Th1 cytokines (eg IFNgamma, IL-1). However, tumors may use numerous mechanisms to adapt to and exploit host and immune-derived cytokines and chronic inflammation responses can promote tumor progression by recruiting Th2 cytokine (eg Il-6, Il-23, TGFb) producing T cells, regulatory T cells and M2 macrophages. Deciphering the key cytokines and cellular factors that determine this balance within the tumor is an ongoing challenge..

B.4. Oncostatin M (OSM)

Oncostatin M is a member of the IL6 cytokine family. The prototype of this family is IL-6, which has been shown to be a central factor in mediating the acute inflammatory response, the transition to chronic inflammation, and the innate immune response.[14] IL-6 is produced relatively promiscuously in tumors by many types of stromal, immune and tumor cells.[15] IL-6 has been considered to promote tumor progression through several pathways including stromal induction of TGFb, immunosuppression, and induction of Epithelial to Mesenchymal Transition (EMT) - a process defined in developmental biology whereby epithelial cells acquire a mesenchymal phenotype and motility.[16] However IL-6 has not emerged as a predictor of response to chemotherapy or endocrine therapy,[17,18] and several clinical trials testing the therapeutic value of anti-IL6 and IL-6 receptor antibodies have had modest results.[19]

Oncostatin M has not been so extensively studied, but may be important in breast cancer. OSM expression appears to be restricted to specific inflammatory cell subsets (macrophages, T-cells and dendritic cells). Evidence that OSM is produced by epithelial cells is inconclusive and unconvincing (see below). OSM signals through two receptor complexes defined by OSMR or LIFR subunits in combination with a common IL-6 family receptor subunit, gp130, that transduces signaling classically through the Jak/Stat3/NFkB pathway, but MAPK and PI3K signaling events also occur.[20] OSM shares some effects with IL-6 on breast cancer cells in-vitro, with enhanced cell motility and invasion reported but also has distinct effects in reducing proliferation and stimulating differentiation and ECM protein production.[21,22] The limited analysis of human tumors to date suggests that an increase in OSM/OSMR can occur in relation to tumor progression in several tumor types[15,23] and that this may be associated with adverse outcome.[24] As described below our recent data also suggests that expression of OSM and its receptor OSMR may be prevalent and important in breast cancer

B.5.S100A7

We and others have identified S100A7 (Psoriasin) as an inflammation-associated protein relevant to breast tumor progression.[25,26] S100A7 is a small calcium-binding protein belonging to

the S100 gene family over-expressed in ductal breast carcinomas, where it associates with aggressive, high grade, ER- tumors, prominent leukocyte infiltration, and poor patient outcome.[27-29] While S100A7 can have both pro- and anti-tumorigenic effects in vitro, it consistently promotes tumorigenesis in vivo based on two breast xenograft mouse models with induction or suppression of expression.[26,30,31] This is consistent with its intracellular mode of action mediated by interaction with Jab1 and stimulatory effects on pro-survival and invasive pathways in breast cells, including PI3K-Akt, NFκB, and AP-1.[32] However some discordance between in vitro and in vivo assays suggests that more careful consideration of the role of the extracellular environment in S100A7 regulation and function is required. S100A7 can be secreted, and by analogy to other S100 proteins, including S100A8, A9, and A12, may regulate inflammatory processes.[33,34] The extracellular action of S100A7 is believed to occur through the receptor for advanced glycation end products (RAGE), and possibly TLR4.[33-35] However discordances in cell type specificity of chemotactic effects identified in two in vitro studies remain to be resolved

C. PRELIMINARY DATA AND OBSERVATIONS
C1. OSM is expressed and OSM/OSMR associates with S100A7+ and ER- status in breast tumors. We initially set out to examine the relationship between cytokines and S100A7 in breast cancer.
- In a case-control cohort of S100A7+ and S100A7- breast tumor specimens, we first observed an association between S100A7 and OSMR mRNA levels by RT-PCR (P=0.0098; Fig 1). However, neither IL-6 nor its receptor (or another IL-6 family cytokine, LIF) correlated with S100A7.
- We next sought to verify these observations by in-silico analysis of a large publically accessible gene expression dataset (UNC microarray database[36]). We confirmed a correlation between S100A7 and OSMR Log2 gene expression ratios (n=179 cases P=0.0002) and also with OSM (P=0.0277). Again, no correlation between S100A7 and IL-6 or IL-6R was observed. We next divided cases into OSM+OSMR+, OSM or OSMR+, OSM-OSMR- groups (or similar based on IL-6 and IL-6R status) on the basis of higher or lower than median expression. There was a clear correlation between OSM/OSMR and both high S100A7 and low ER expression that was lacking with IL-6/IL-6R (Fig 2). Importantly, even within the ER+ subset, tumors positive for either OSM or OSMR have significantly lower levels of ER (P=0.04), consistent with an effect of OSM/OSMR on ER. Thus, ties between ER, S100A7, and OSM signaling, but not IL-6 signaling, emerged from both direct and in-silico analysis.

C2. OSM/OSMR pathway and S100A7 correlate with poor outcome. We next analyzed the relation between OSM/OSMR and S100A7 and outcome.
- In the UNC dataset patients within the same OSM/OSMR +/+, +/-, and -/- subgroups defined above manifested 5 year disease free survival (DFS) rates of 25%, 37% and 68% respectively (Fig 3a).When these three patient subgroups were further subdivided into S100A7 high/low groups, S100A7 correlated with reduced overall patient survival only in the OSM&OSMR+ subgroup (HR=2.83, P=0.0371, Fig 3b). This observation still holds when one considers only ER– tumors (HR=2.96, P=0.0729, n=28), suggesting that this association is not simply due to the relation between S100A7 and ER– status. In contrast, S100A7 had no prognostic significance when patients were divided according to IL-6 and IL-6R status.

C3. OSM downregulates ER, upregulates S100A7, and promotes cell migration in a S100A7-dependent manner in-vitro. We next determined if OSM has a direct effect on S100A7 and ER in breast cell lines.

- It has been previously noted that OSM influences ER expression[37] but this has not been pursued. We verified this observation using both MCF7 and T47D cells, which show a reversible ~60-70% reduction in ER mRNA levels after 24 hours of OSM treatment (Fig. 4). IL-6 also reduced ER expression but not to the same extent as OSM (~40-50% reduction). We then determined the effect of OSM on S100A7 in MCF7 cells (Fig. 5), finding that OSM is a potent inducer of S100A7 mRNA and protein expression in both ER+ (MCF7, T47D) and ER- (MDA-MB-468) breast cancer cell lines, despite high endogenous levels of S100A7 in the latter cell line.

- We have initially pursued the involvement of specific signaling cascades and the functional significance of S100A7 induction. Selective inhibition of the ERK1/2 MAPK (by UO126) has a modest impact on S100A7 induction (Fig. 6a), but this could be attributable to partial coincidental STAT3 inhibition since overexpression of a dominant negative (Y705F) STAT3 mutant can substantially reduce S100A7 induction (Fig. 6b). PI3K/AKT inhibition (by LY294002) has minimal effect on S100A7 induction. OSM is already known to stimulate a migratory/invasiveness phenotype in MCF7 cells.[21,38,39] RNAi inhibition of S100A7 demonstrates that S100A7 is necessary for this phenotype (Fig. 7). Similar but diminished effects on migration and requirement for S100A7 was also seen with IL-6 (not shown).

C4. ER- and S100A7+ status correlate with inflammation and distinct profiles of intra-tumoral immune response in breast tumors

- To examine further the relation between inflammation and S100A7 and ER in-vivo we studied data obtained from a 424 case TMA designed to mirror the distribution of major prognostic clinical-pathological features (eg T size, N stage, grade and ER status) in the MBTB.[40,41] Inflammation was associated with S100A7+ status (in 17%, 44% and 56% of tumors categorized as having low, moderate, or high inflammation by semi-quantitative H&E assessment, p<0.0001) and inversely with ER levels (p<0.0001).

- We then examined the relation between S100A7 and specific intra-tumoral leukocyte subsets in data from tumors with marked T cell infiltrates in an overlapping set of ER- cases (n=255). Surprisingly, in assessing the immune cell composition in the subgroup of tumors with highest levels of chronic inflammation (CD3+ T cells greater than the median), S100A7 was negatively correlated with CD3 (P=0.0079), CD8 (cytotoxic T cell lineage; P=0.0257), CD25 (T cell activation marker; P=0.0240), and TIA1 (cytotoxic T cell secretory granule protein; P=0.0369) (Table 1). Furthermore, high levels of TIA1+ cells predicted for good DFS only in S100A7+ tumors (HR=0.36, P=0.0084, Fig. 8).

- It has been reported that OSM can be produced by activated T cells and monocytes[42,43] while many cells are responsible for producing IL-6. This is supported by analysis of the UNC dataset, which shows that OSM correlates only with CD25 (T cell activation marker; P=0.0220) and strongly with CD68 (monocytes/macrophages; P<0.0001), while IL-6 correlates with several markers of T cells, B cells, monocytes, mast cells, and neutrophils.

- We also re-examined ER– OSMR+ subsets in the UNC dataset and found S100A7 has the strongest prognostic relevance. Also consistent with our TMA observations we found that S100A7+ cases had significantly lower expression of CD3 and CD8, but not CD4 (T helper

cells) (Fig. 9). In sum these data imply a relation between non specific inflammation and ER- and S100A7+ status, and a relation between S100A7 and immuno-suppression indicated by reduced activated cytotoxic T cells.

D. RESEARCH PLAN

The overall tenet is that inflammation and the associated immune response influences tumor biology and progression.[44-47]

The **HYPOTHESIS** is that OSM derived from intra-tumoral inflammation suppresses ER and promotes invasion in breast tumor cells and that S100A7 is linked to the mechanism of action and promotes this type of inflammation response.

The **SPECIFIC AIMS** are to address the following questions that stem from this hypothesis;

1) What is the mechanism of OSM suppression of ER and induction of EMT-like properties? We will manipulate the putative components of these pathways in breast cell lines using inhibitors, RNAi and cell transfection approaches to delineate the role of S100A7 and specific kinase and signaling pathways.

2) What is the in-vivo evidence that the OSM/OSMR/S100A7 pathway is involved in breast tumor progression? We will determine the cellular origin of OSM and cellular targets defined by expression of the OSMR and examine the relation between OSM/OSMR/S100A7 and response to adjuvant endocrine therapy in breast tumors.

3) What is the effect of S100A7 on the inflammatory response in breast tumors? We will examine the effect of recombinant S100A7 on inflammatory and immune cells in-vitro and examine the effect of S100A7 on the intra-tumoral inflammatory response in an in-vivo xenograft model.

AIM 1. What is the mechanism of OSM suppression of ER and induction of EMT-like properties in breast tumor cells ?

We have shown that OSM upregulates S100A7, downregulates ER, and exerts effects on a single aspect of an EMT-like phenotype (migration) that requires S100A7. It is possible that the effects of OSM on these parameters occur through independent signaling pathways diverging from the established signaling intermediates of OSMR (STAT3, PI3K/AKT, or ERK). It is also plausible that S100A7 plays a role as a necessary factor in both ER suppression and EMT induction. Finally it is possible that ER suppression is a consequence of EMT. We will delineate the pathways by manipulating signaling and gene expression in cell lines in-vitro and in-vivo, initially focusing on ER and EMT issues separately.

In-vitro studies focused on ER issue: OSM and, to a lesser extent, IL-6, can suppress ERα mRNA expression in-vitro in MCF7 and T47D cells. We will validate these observations using additional ER+ cell lines including ZR75, SKBR3, and BT474. ERα expression will be assessed by both western blot and RT-PCR to determine if cytokine treatment affects both mRNA and protein expression. We will perform dose-response assays ranging from 1 to 200 ng/ml to determine the minimum and also the optimum cytokine concentration for ERα suppression. We will address the kinetics of ERα suppression by performing time-course assays (minutes to several days) and will assess the persistence of suppression and rate of ERα recovery following cytokine withdrawal. Because OSM can signal through the OSMR or LIF receptors we will knock down OSMR or LIFR expression separately using siRNA to verify the role of OSMR in ERα regulation. Our data suggests that LIF expression is low (Fig. 1) and LIFR is not detectable

(not shown) in-vivo in tumors, but engagement of LIFR might occur in cell lines and influence interpretation of in-vitro experiments.

It is possible that ERα suppression may be a direct result of altered MAPK, PI3K/Akt activities and phosphorylation changing ER protein stability,[48] or an indirect result of S100A7-Jab1 induction leading to increased NFkB activation,[32] or enhanced degradation via other COP9 signalosome mediated effects on proteasome degradation,[49] or a result of EMT driven changes in NFkB.[50-52] Our data (Fig. 4) suggests that transcriptional downregulation of ER is partly involved, perhaps downstream of NFkB.

To distinguish these possibilities the three principle signaling pathways downstream of the OSM receptors (STAT3, PI3K, and the ERK, p38, and JNK MAP kinases[20]) will be interrogated using small molecule kinase inhibitors or, for STAT3, overexpression of a dominant negative mutant (Y705F, as in Fig. 6) to delineate the relation with ER suppression. We will also assess ERα mRNA and protein levels to determine the nature of suppression, as well as delineating the time course and requirements for this effect of S100A7. We will knockdown S100A7 expression by RNAi during cytokine treatment and monitor ERα mRNA and protein expression and ER responsive genes PR and pS2. In addition, we are in the process of generating MCF7 clones using a TET-ON inducible S100A7 expression system to determine if S100A7 can affect ERα in the absence of cytokine treatment. If S100A7 is found to influence ERα, we will perform further experiments to delineate if this is a direct effect through the previously characterized S100A7-Jab1 pathway[32] by siRNA Jab1 knockdown and downstream AP-1 and NFkB activity analysis by reporter gene and DNA binding assays. We will also measure ERα phosphorylation at several sites including phosho-serines 118 and 167 thought to be targets of the kinase pathways and important in regulating ER turnover in breast cancer[48,53,54] and would anticipate absent or delayed effects if ER suppression is attributable to S100A7-Jab1-signalosome effects. The role of common endpoints for these pathways (NFkB activation and proteasome function) will be tested using the IKK inhibitor BAY 11-7082 and Velcade as we have previously described.[12]

In-vitro studies focused on EMT issue: It is important to examine the role of OSM in mediating the EMT-like phenotype in parallel with our main focus on ER because these processes may be linked.[55] The master EMT regulator Snail can directly suppress ER expression[56] and conversely ER may suppress EMT regulators.[57,58] We and others have shown that OSM can induce an EMT-like phenotype involving enhanced migration and altered differentiation that might lead to ER loss. OSM action in MCF7 cells may be similar to IL-6 (which induces EMT regulators snail and twist, suppresses E-cadherin, and induces N-cadherin and vimentin). We will examine the effect of OSM in the breast cell lines listed above on these central regulators and biomarkers of EMT by western blot and RT-PCR analysis. If we observe activation of EMT pathways we will determine the timing of these changes relative to ER suppression and if EMT regulators are required to suppress ER directly or through S100A7.

In-vivo studies. To establish the contribution of OSM/OSMR to the pathways leading to ER suppression in-vivo we will manipulate OSMR in breast cell line xenograft models. While human MCF7 cells are most widely used for in-vivo hormone response studies and there are MCF7 cell models where acquired resistance is associated with loss of ER,[10,59] unknowns that would need to be resolved with pilot experiments include the presence of an OSM expressing

inflammatory response in nu/nu mice (lacking T cell mediated response but with relative preservation of macrophages and innate immune responses), the possibility of intrinsic S100A7 induction in tumor xenografts, and confirmation that mOSM can signal through hOSMR as has been reported.[60] Subject to resolving these variables we will examine the effect of OSMR on ER and EMT features in-vivo by stable transfection to create inducible TET-ON OSMR and shRNA OSMR knockdown MCF7 cells. Following clonal selection and characterization of expression, wild type and gene transfected cells will be used to establish xenografts in nu/nu mice in the presense of implanted estradiol pellets and using methodology with which we are familiar.[32] When tumors reach 150 mm3 (~5 wks) subgroups will receive ICI 182,780 (faslodex) (and +/- doxycycline for TET-ON cells) and effects measured by monitoring a) in-vivo growth and b) tumor histology and ER gene expression as detailed above, subsequent to harvesting and after development of endocrine resistant growth. We would predict that enhanced OSMR signaling would promote ER suppression and may facilitate onset of resistance while knockdown of OSMR would prevent ER suppression and resistance. If S100A7 is shown to influence ER suppression we will repeat these in-vivo experiments with TET-ON S100A7 MCF7 cells to compare with OSM/OSMR effects.

AIM 2. What is the in-vivo evidence that the OSM/OSMR/S100A7 pathway is involved in breast tumor progression? We will address this through three subaims as follows;
A). **Determination of the cellular origins of OSM/OSMR expression in breast tumors.**
Previous work has shown that OSM is produced by a limited range of inflammatory cell types (T cells and macrophages), but evidence that OSM is produced by tumor cells is inconclusive. OSM is not detected in breast cell lines in-vitro and a single study suggesting expression in-vivo in breast cancer was restricted to IHC determination of protein signal rather than mRNA expression.[23] Two further studies in prostate[61] and ovarian[62] cancer suggest that OSM is expressed in tumor cells. OSMR may be expressed in stromal cells and only in some tumor cells,[61] and a short form has been described in lung cancer.[63] In all studies biological conclusions around OSM are limited because of issues related to restriction to IHC signal specificity (or whole tissue RT-PCR) and lack of discrimination of stromal cell types. Clinical conclusions are limited by small study size and biased composition.

To determine the cellular origins of OSM and localization of OSMR in breast tumors an expression localization cohort will be selected using the following criteria to populate subgroups anticipated to exhibit high and low OSM/OSMR expression (ER+/- and Inflammation high/low as described above, to create 4 subgroups, n=12 each). A TMA will be constructed and used to develop IHC protocols and then to delineate OSM and OSMR expression by IHC. Subsets of cases that demonstrate high, medium, low/negative expression of OSM and OSMR will be selected for study by ISH on whole sections from paraffin blocks and RT-PCR and Western blot conducted on extracts from frozen blocks to correlate and establish IHC specificity. Cellular origins of OSM/OSMR expression in relation to tumor and fibroblast and inflammatory cells will be determined by IHC for T cells, B cells, macrophage subsets (CD3, CD4, CD8, TIA1, CD25, FoxP3, CD68, CD20) and tumor cells (cytokeratins) on serial sections (see below). If necessary laser microdissection will be performed prior to mRNA and protein extraction to further refine cellular compartments. We have extensive experience in antibody validation for IHC and in gene expression analysis by all approaches including ISH and microdissection of breast tumors.[27,63,64]

B) **Determination of the association of OSM/OSMR with molecular subtypes of breast cancer.** Our preliminary data suggests that activation of the OSM/OSMR pathway contributes to suppression of ER and the pathway is linked to subsets of breast tumors defined by S100A7 and specific intra-tumoral inflammatory responses. Others have identified activation of Growth Factor pathways as important in ER suppression.[4,65,66] However in reassessment of our TMA424 cohort data described above (defining growth factor + status on the basis of Her2+ and/or EGFR+ expression), while S100A7 was more frequent in ER+/GF+ than ER+/GF- tumors (10/30=33% vs 36/240=15% p=0.019), S100A7 was equally distributed in ER-/GF+ and ER-/GF- tumors (66% vs 48%). This supports the notion that the putative OSM/OSMR/S100A7 pathway to ER suppression may be different but exists within overlapping patient groups. We will therefore validate our observations from aim1 in-vivo, and explore the possibility that the OSM/OSMR cytokine pathway driven ER suppression is a distinct mechanism from Growth Factor driven ER suppression, Since the TMA424 is now exhausted, a 180 case molecular subtype cohort will be selected using the following criteria to populate subgroups representing different ERα and growth factor status (ER high/low/- and GFR high/low/- invasive ductal cases, x9 groups, each n=20). ER categories will correspond to clinical Allred scores of >3, 3-2, and 2-0) and GFR categories will correspond to Her2 3&2+, 1+, 0. Further refinement of GFR status will be achieved on the basis of EGFR levels determined by IHC after TMA construction.

We will construct a TMA and stain, score, and assess gene expression for ER, Her2 and EGFR (to confirm categorization within regions cored) and then OSM/OSMR, Stat3/MAPK, S100A7, NFkB, p63/p52, ER to address three issues;
a) We will examine the status of the OSM/OSMR pathway within these molecular subclasses for evidence that suppression of ER related to OSM/OSMR is a distinct mechanism to suppression related to GF activity. We would expect only partial overlap with GF receptors within ERlow and ER- categories. Staining will be conducted on semi-serial sections to facilitate correlation of gene expression between cell regions and components. Validation of some IHC biomarker assays will be done on the cohort in 2A where needed using the same approach.
b) We will determine in-vivo correlations in support of functional relationships defined in aim1. Dual staining protocols for OSMR/ER and OSMR/S100A7 will be developed as indicated to directly assess cell specific co-localization..
c) We will examine the relation between S100A7 and the pattern of inflammation as defined by inflammatory and immune cell types and their expression of OSM.

C) **Determination of the significance of OSM/OSMR in relation to endocrine therapy.** Our data suggests that activation of the OSM/OSMR pathway in primary tumors predicts for suppression of ER with tumor progression and reduced effectiveness of adjuvant endocrine therapy (TAM). We will do this by comparing +/- OSM activation in ER+ breast cancers treated with only TAM and in cancers treated with chemotherapy and TAM, to see if any observed inferior outcome in the OSM activated group is prevented by treatment with chemotherapy. That is, whether ER+ OSM activated cancers behave more like ER- breast cancers in their response to treatment. A 1000 case outcomes cohort will be assessed for OSM/OSMR/S100A7, ER/PR/ and Her2/EGFR expression (as previously described). The cohort will be selected randomly from a larger set selected first using the following criteria: cases from 1989-1999 and >10 years followup data, ER+ invasive breast cancer, treated with breast conserving surgery or mastectomy. Patients included may have received RT as part of locoregional treatment, with

adjuvant systemic therapy with endocrine therapy alone or endocrine+chemotherapy. We will exclude inflammatory breast cancer, cases with distant metastasis, unknown nodal status, or a previous or synchronous breast cancer. Initial query of the MBTB with the above criteria has identified 953 and 330 cases treated with endocrine and endocrine+chemotherapy respectively from which to construct a TMA.

We recognize that only if OSM/OSMR emerged as a very strong predictive factor would it be likely to have a clinical impact on selection for endocrine therapy. However, it is possible that the OSM/OSMR pathway might influence outcome in patients treated by hormones and chemotherapy through other mechanisms (eg EMT properties leading to ER supression as considered in aim1), in which case a value as a biomarker would warrant further examination. If the above studies consolidate our hypothesis we would pursue prognostic or predictive value further by applying to study the NCIC-CTG MA-14 trial cohort (a randomized trial of antiestrogen +/- octreotide) with ~110/330 events in patients in each arm) focusing on the antiestrogen alone arm. These studies will lay the groundwork to justify possible future intervention trials with anti-inflammatory therapy in conjunction with endocrine therapies for ER- tumors.

Cohort Sources & Methods: We will utilize two tissue sources – the BCCA Tumor Tissue Repository (TTR) prospectively accrues tissues and blood samples from breast cancer patients (>1100 cases) and the Manitoba Breast Tumor Bank (MBTB) is an older biobank that collects tissues from ER/PR assay samples (>3500 cases), both directed by PHW). Biobanking processes at both sites are under REB approved protocols and governed by SOP procedures. Both process breast specimens into matching frozen and paraffin blocks and annotate biospecimens with composition, diagnosis, treatment and also outcomes data derived from provincial cancer registries. The TTR source will be used to delineate the cellular origins and relation between OSM/OSMR and pathway components in whole sections and to validate antibodies. This is because it has biospecimens with central and peripheral tumor and adjacent normal tissue representation and our laboratory has close access for possible microdissection. The MBTB biospecimens are predominantly representative of only central tumor regions but have extensive outcomes data and will be used to address clinical outcomes questions.

Scoring will be conducted using semi-quantitative systems in both tumor and stromal compartments. A modified H score approach will be used for most markers [IHC score = (% positive) x (staining intensity 0 to 3) = 0-300] and several empirical cutpoints assessed as defined by percentile scores. Inflammatory cells will also be quantified by Chalkley grid approach within tumor epithelium and stromal compartments. Data on patient demographics, pathologic T and N staging, histologic subtype, grade, lymphovascular invasion, locoregional and systemic therapy, patterns of relapse, relapse free survival, and overall survival will be abstracted. Analysis will include tests for correlations between biomarkers and tumor prognostic parameters such as grade, stage and T cell infiltrates, relapse free and overall survival using Spearman, t-tests, and log rank tests (GraphPad Prism software). Multivariable analyses will be performed to examine the prognostic significance of the biomarkers on relapse free and overall survival.

AIM 3. What is the effect of S100A7 on the inflammatory response in breast tumors?

Rationale. We have shown that OSM induction of S100A7 can occur in-vitro and that a relationship exists between OSM, S100A7, and specific patterns of intra-tumoral inflammation in-vivo. It is therefore possible that the associations observed in-vivo relate to direct effects or that the intrinsic activation of S100A7 by factors other than OSM lead to either a) secretion of S100A7 that can exert immunomodulatory actions that may promote OSM expression or b) stimulation of the S100A7-Jab1 pathway in breast tumor cells and induction of NFkB/AP1 regulated cytokines in tumor cells that indirectly regulate OSM expression in the inflammatory response.[67] To explore these possibilities we will examine the effects of S100A7 on leukocyte chemotaxis in-vitro and on intra-tumoral inflammatory responses in-vivo.

A) Effects of S100A7 on immune cells in vitro. Previous reports have identified S100A7 a chemotactic factor, but divergent results have suggested S100A7 action is either specific for CD4+ T cells and neutrophils, or a broader non-specific effect on lymphocytes, and monocytes, as well as neutrophils. These effects may be mediated through the RAGE or TLR4 receptors. However our own TMA data noted above shows a negative correlation between S100A7 and CD8 T cells in breast tumors within ER- tumors that have intrinsically high inflammation (Table 1). Thus, the in-vivo relevance as a chemotactic factor is an outstanding issue.

We will examine chemotaxis using recombinant S100A7 (generated and validated by crystallography with our collaborators).[68] We will also use alternate sources of rhS100A7 (available through our collaborator S. Yuspa[69] and commercial sources Abnova) and control proteins to test the effects of direct administration on leukocytes and to resolve discordances in previous studies that may be due to bacterial contaminants. We also will employ several breast cell lines that constitutively overexpress endogenous S100A7 (MDA-MB-468) or transgenic S100A7 (MDA-MB-231 and MCF7 TET-ON cells under development, see above) to generate a panel of S100A7 +/- conditioned media.

T cells, monocytes and neutrophils will be isolated from peripheral blood from healthy volunteers. Neutrophils will be purified using dextran-ficoll sedimentation, and monocytes and T cells isolated using a BD InFlux fluorescence activated cell sorter (FACS). Leukocytes will be tested in transwell migration assays using fibronectin-coated (for T cell migration) 5 μm polycarbonate filters. Chemo attractants will include rhS100A7 (0.1 to 3000 ng/ml, diluted in RPMI) or conditioned media generated by S100A7 inducible cell lines (+/- induction) and S100A7 constitutive cells (+/- Jab1 knockdown). Assay duration will depend on the type of leukocyte being examined (neutrophils 1hr, monocytes 1.5hr, T cells 3hr). Known chemotactic factors for each cell type will also be used as positive controls for cells (IL-8 for neutrophils, MCP-1 for monocytes, and CCL5/RANTES for T cells). Known chemotactic targets for S100A7 will be used as controls for rS100A7 reagents (human keratinocytes). Additional controls to address potential LPS contamination of the rhS100A7 will include polymyxin B (an LPS inhibitor) in stimulation assays and heat-inactivation in separate control assays. Cells attached to the lower filter surface will be stained with a vital blue stain and counted using a chalkley grid approach and standardized to media only controls. To distinguish between intracellular (Jab1 mediated) and extracellular (RAGE and TLR4 receptors)[35] actions, we will use Jab1 knockdown (as we have previously published[41]) in conjunction with S100A7 induction to generate media and RAGE and TLR4 blocking antibodies.

If we observe effects then chemotaxis (directional migration) versus chemokinesis (intrinsic random migration) effects will be determined by adding rhS100A7 to both the lower and upper assay chambers. We will also examine leukocytes for possible induction of OSM expression. In future studies we will proceed to explore effects of S100A7 on CD4+ and CD8+ T cell activation, proliferation, and polarization and CD8 cytotoxicity.

B) Effects of S100A7 on intra-tumoral inflammatory responses in vivo. To test the effect of S100A7 on intra-tumoral inflammatory and anti-tumor immune responses in vivo we will manipulate S100A7 expression in a murine breast tumor cell line model and xenografts. A reproducible inflammatory response is induced in this model by adoptive transfer of CD4+ and CD8+ T cells targeting a model antigen epitope expressed by the tumor cells.[70]

We will transfect NOP breast cells[70] with mS100A7 to avoid issues with immunogenicity with the human protein. We have previously shown that mS100A7 is structurally highly similar to hS100A7 and exhibits similar patterns and regulation of expression to that of hS100A7 in the context of both skin inflammation and breast tumorigenesis.[71] It will be important to determine if the NOP lines have endogenous mS100A7 expression or if mS100A7 is induced with tumorigenesis in vivo. We will test this by RT-PCR on cell lines cultured in vitro and also frozen samples of xenograft tumors derived from them. If no endogenous mS100A7 expression is detected, we will stably transfect NOP cells to overexpress mS100A7. If we observe endogenous mS100A7 expression, we will initiate the alternative strategy using shRNA vectors to generate stable mS100A7 knockdown clones. We would initially select NOP23 cells that have been well characterized and exhibit reproducible partial responses to T-cell challenge which would facilitate monitoring of the subsequent inflammatory response.

We will use the transfected cell lines with different S100A7 expression levels and parental controls to perform tumorigenesis assays by injecting tumor cells bilaterally at a dose of ~1×10^6 cells into the mammary fat pads of immunocompetent female mice C57BL/6 mice. As previously described[70] once tumors reach ~50mm2, mice will receive mice OT-1 and OT-II T cells by adoptive transfer (expt day 0). Tumor diameters will be measured at weekly intervals thereafter until termination of the experiment (at 3 weeks). Subgroups of animals will be sacrificed at day 0, 5, 7, 10 and 20. Primary tumor histology and intra-tumoral inflammatory and immune infiltrates will be characterized for host and donor cells and compared between experimental groups of mS100A7-expressing tumors versus parental tumors. S100A7, OSM and OSMR and LIFR (important in murine OSM signaling[60]) expression will be assayed by IHC together with the lymphocyte markers CD3, CD4, CD8, CD20, CD25, GzmB, and FoxP3, as well as the macrophage marker F4/80 and the neutrophil marker Ly6G.

We will also characterize the activation state of tumor-infiltrating lymphocytes (TIL). Portions of the freshly excised tumors will be disaggregated and passed through a 40 um membrane for TIL isolation. Flow cytometry will be used to quantify CD4 and CD8 T cells expressing the activation/differentiation markers CD25, GzmB, and FoxP3, as well as the polarization state-specific cytokines listed above. Finally, FACS-isolated T cells within the TIL population will be activated in vitro using plate-bound anti-CD3 antibodies to identify the presence or absence of activation defects with respect to the tumor mS100A7 expression status.

In the future, if tumor expression of mS100A7 is found to be associated with changes in the murine anti-tumor immune response we would examine S100A7 effects in the context of other NOP cell lines that exhibit different intrinsic sensitivities to T cells. We would also determine if the effect is mediated by intra- or extracellular mS100A7 activity. We would verify that mS100A7 has similar intracellular effects to hS100A7 by assaying for intracellular S100A7-Jab1 interaction and downstream effects (p27 downregulation, AP-1 and NFkB upregulation (using luciferase reporter gene assays), and nuclear localization of Jab1.[30,32] We would then seek to abrogate mS100A7 binding with Jab1 by generating cell clones with mutation at a key residue (Glu60) in the Jab1 binding site, which we have shown to be necessary for Jab1 interaction in humans.[32]

E. TEAM & TIMELINES
The PI has published experience in study of molecular biology of breast cancer, S100A7 and ER biology, and with all models and most assays described. The co-PIs and collaborators contribute complimentary expertise in cancer immunology (BHN), endocrine therapy (NM), outcomes analysis (PT) and ER biology (LCM). Aim1 is a continuation of ongoing studies and in-vitro studies will be complete by 36mths and in-vivo studies by 60mths. Aim2 will be initiated at mth6 and will complete by 36mths. Aim3 will be initiated at 24ths and will complete at 60mths.

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